ACINETOBACTER SPECIES IN THE HOSPITAL ENVIRONMENT

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Tracing and epidemiology

Acinetobacter species in het ziekenhuismilieu
Opsporing en epidemiologie

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CHAPTER 1

INTRODUCTION

In the past decades, bacteria of the genus *Acinetobacter* have increasingly been associated with nosocomial infection [1]. Acinetobacters are Gram-negative coccobacilli with a strictly aerobic metabolism. The bacteria are nonmotile, oxidase-negative and catalase-positive. They are widespread in nature and have been isolated from soil and water and from a variety of samples from human and animal sources [6]. In general, acinetobacters do not seem to be particularly virulent. They have, however, been involved in severe infections, e.g. pneumonia, meningitis and septicaemia, especially in seriously ill hospitalized patients [1, 5]. In recent years, many hospital outbreaks by acinetobacters have been reported [1]. Most outbreaks occurred in surgical departments or intensive care units. The strains involved are often resistant to antibiotics. In these cases the infections are difficult to treat [2] and may be fatal. The sources and mode of transmission of acinetobacters in the hospital environment are not well understood.

The genus *Acinetobacter* is heterogeneous and has a complex taxonomic history. Bacteria, now allocated to this genus, have been described by different names, including *Micrococcus calco-aceticus*, *Herellea vaginicola* and *Moraxella lwoffii*. In the first edition of Bergey's Manual of Systematic Bacteriology all strains of *Acinetobacter* were considered to be members of a single species, *A. calcoaceticus*, because criteria for the differentiation of several species were not satisfactory [7]. In recent years, different groups within the genus *Acinetobacter* were identified by the use of DNA-DNA hybridization [3, 4, 10]. Four new species were proposed, *A. baumannii*, *A. haemolyticus*, *A. johnsonii* and *A. junii* and the descriptions of *A. calcoaceticus* and *A. lwoffii* were emended. Another species, *A. radioresistens*, was proposed on the basis of genetical and enzymatical properties of strains [8]. Strains of a number of the DNA-hybridization groups (genospecies) may be identified phenotypically by the use of a system of multiple biochemical tests [3]. This system is, however, not yet currently applied. In clinical microbiology, acinetobacters are often identified as *Acinetobacter* species and merely distinguished in glucose-acidifying strains, sometimes designated as the (obsolete) species *Acineto-*

bacter anitratus, and in glucose non-acidifying acinetobacters, often designated as *A. lwoffii* [9]. In the present thesis, acinetobacters are designated as belonging to the species *A. calcoaceticus sensu lato* [7], except for chapter 7, which deals with the recently described DNA hybridization groups [3, 10].

The purpose of the present investigation was to elucidate the niches and the mode of spread of *Acinetobacter* strains in the hospital environment. For this purpose, typing methods were needed which allowed the tracing of epidemical strains. Although several typing methods had been described [1], no current method was available at the beginning of the study. Therefore, two typing methods were developed and tested on strains, which were collected during the investigation. Subsequently, studies were performed which were focused on the epidemiology of the bacteria in the hospital environment. The studies were aimed to answer the following questions:

- Which methods can be used to type *Acinetobacter* strains and how useful are the methods in hospital epidemiology?
- Where do the strains reside in patients and in the environment and, how are the strains transmitted?
- Are the cell envelope protein profiles, which are used to type strains, also fit for identification of strains according to the recent classification scheme of DNA groups?
- To which DNA groups do *Acinetobacter* strains, involved in hospital infections and epidemics, belong?

In an initial study, the usefulness of cell envelope protein sodiumdodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) as a typing method was explored. The results are presented in chapter 2. In a next study, described in chapter 3, this method was applied to investigate the presence of acinetobacters in a number of body sites of patients over a period of time. During two outbreaks in intensive care units *Acinetobacter* strains were traced. The dissemination of epidemic strains among patients and in their environment is analysed in the chapters 4 and 5. The usefulness of an additional typing method, based on the growth of strains on carbon sources, is evaluated in chapter 6. In cooperation with other research groups, the relation between cell envelope protein profiles and DNA hybridization data of strains was investigated. The study is discussed in chapter 7. Finally, in chapter 8, the complete investigation is considered and findings are related to data from literature.

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CHAPTER 2

CELL ENVELOPE PROTEIN PROFILES OF *ACINETOBACTER CALCOACETICUS* STRAINS ISOLATED IN HOSPITALS

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SUMMARY

The cell envelope protein patterns of 78 strains of *Acinetobacter calcoaceticus*, mainly isolated in hospitals, were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The patterns were stable and reproducible. Comparison of the protein profiles made possible differentiation between two groups of strains. The patterns of the first group could be classified on the basis of concordance. Some profiles appeared to be associated with the epidemiological origin of the strains. The second group consisted of strains with unique patterns which could not be classified. Comparison of SDS-PAGE patterns appears to be a suitable method for the relative classification of *A. calcoaceticus* strains of nosocomial origin.

INTRODUCTION

Despite its low virulence, *Acinetobacter calcoaceticus* has been described repeatedly as a cause of hospital infections (Glew *et al.*, 1977; Ramphal and Kluge, 1979; Holton, 1982; Sherertz and Sullivan, 1985). To study the mode of transmission in hospitals, a classification system is needed by which identical strains may be recognised. Various methods of classifying *A. calcoaceticus* have been described (Marcus *et al.*, 1969; Alexander *et al.*, 1984; Das and Ayliffe, 1984) but none appears to be in general use. In the absence of usable

classification methods, isolates of *A. calcoaceticus* are often merely divided into two groups on the basis of their saccharolytic capacity (Juni, 1984). Acid-forming strains are referred to as "var. *anitratus*", non-acid-forming strains as "var. *lwoffi*" (Rosenthal, 1978).
Protein patterns of cell membranes of Gram-negative bacteria obtained by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) can be used to identify strains and clones (Overbeeke and Lugtenberg, 1980; Achtman *et al.*, 1983) and this approach has been used in epidemiological studies (Loeb and Smith, 1980; Frasch and Mocca, 1982)
The purpose of the present study was to investigate whether SDS-PAGE protein patterns could be used to classify strains of *A. calcoaceticus*. We investigated (i) whether strains of this species have characteristic and reproducible protein patterns when cell envelopes are subjected to SDS-PAGE, (ii) whether epidemiologically related strains have similar and unrelated strains dissimilar patterns, and (iii) whether a relationship exists between protein patterns and such easily detectable properties as acid production from glucose and haemolysis on sheep blood agar. For this purpose, 78 strains were selected from culture collections, clinical specimens, the skin of volunteers and the inanimate environment.

MATERIALS AND METHODS

Selection and sources of strains of A. calcoaceticus

The following reference strains were studied: ATCC 23055 (type strain of *A. calcoaceticus*, Beijerinck 1911), ATCC 15309 (type strain of *A. lwoffi*), ATCC 19639 (original strain Beijerinck), ATCC 13809, ATCC 9957 and NCTC 7844. Other strains included in the study were Gilardi 2890, a strain originally obtained from B. Vogel (Basle) and four strains from the Microbiological Laboratory of Delft Technical University, designated LMD 70.9, 79.41, 81.109 and 82.54. All the other strains were isolated in the Netherlands during the period 1981-1985, mainly from hospital environments. Twenty-eight strains were isolated at the University Hospital Rotterdam (23 from patients, 3 from healthy volunteers and 2 from the environment). The remaining 38 strains were isolated from patients in smaller hospitals in Rotterdam and Dordrecht and in the University Hospitals of Leiden, Utrecht and Nijmegen. Four strains were cultivated from soil. During the period of study there were episodes of increased isolations of *A. calcoaceticus* in the University Hospitals in Rotterdam and Utrecht. The strains were kept at - 70°C in broth containing glycerol 10% v/v or in a lyophilised state.

Identification of strains

Before the isolation of cell envelopes, all the strains were identified as *A. calcoaceticus* on the basis of the following characteristics (Cowan, 1974): Gram-negative coccobacilli, nonmotile (by hanging-drop method), catalase-positive, oxidase-negative (Kovács technique), non-fermenting and in general incapable of nitrate reduction. As a supplementary test the hydrolysis of Tween 80 was investigated (Sierra, 1957). All the strains gave positive results in this test, though quantitative differences were found. The strains were differentiated into saccharolytic and asaccharolytic varieties on the basis of aerobic acidification of glucose (Hugh, 1978). The capacity to produce haemolysin on Blood Agar (CM 55, Oxoid, with 5% sheep blood) was also investigated. All the media were incubated at 30°C except for the broth for the motility study, which was incubated at room temperature (Hugh, 1978). Motility, reaction with Gram's stain, oxidase and catalase production, and haemolysis were evaluated after 20 h, the other tests after 48 h.

Media and growth conditions

For the isolation of cell envelopes, stored strains were subcultured on blood agar. Various colonies of the same morphological type were inoculated into Nutrient Broth No. 2 (CM 67, Oxoid) and incubated at 30°C for 20 h with vigorous aeration.

Isolation of cell envelope fractions

The isolation of cell envelope fractions and the preparation of samples for electrophoresis were performed by the method described by Lugtenberg *et al.* (1984). Briefly, cells from 20-h cultures were resuspended, after centrifugation, in 5 ml of 50 mM Tris(hydroxymethyl)aminomethane-HCl 2mM ethylene-diaminetetraacetic acid (EDTA), *p*H 8.5. The cells were disintegrated by ultrasonic treatments of *c.* 20 s three to six times, with cooling (100 watt ultrasonic disintegrator; MSE, Crawley, Sussex). The cell debris was centrifuged for 20 min at 900 *g*. The supernate thus obtained was centrifuged for 60 min at 12 300 *g*. According to the quantity obtained, the pellet containing the cell envelopes was resuspended in 100-200 µl of 2 mM Tris-HCL, *p*H 7.7.

Polyacrylamide gel electrophoresis

The proteins were separated electrophoretically and stained by the procedure of Lugtenberg *et al.* (1975) with the following modifications. A Protean II 16 x 16 cm slab cell apparatus (Bio-Rad, Richmond, CA, USA) was used for electrophoresis. The slabs had a thickness of 1.5 mm and 25 μl of a sample were applied per slot. Electrophoresis was performed at constant currents of 30 mA and 35 mA for the stacking and the running gel respectively. Of the three gel systems tested (A, B and C - Lugtenberg *et al.*, 1984), system A mostly gave the best separation. This system was, therefore, used routinely. The mol. wt standards were phosphorylase B (97 400), bovine albumin (66 000), ovalbumin (45 000), glyceraldehyde-3-phosphate dehydrogenase subunit (36 000), carbonic anhydrase (29 000), trypsinogen (24 000), trypsin inhibitor (20 100) and lactalbumin (14 200) (Sigma Chemical Co., St Louis, MO, USA).

RESULTS

General characteristics and stability of cell envelope protein patterns

The patterns of almost all the strains were characterised by one deeply stained band with an apparent mol. wt of (39.8-46.8) x 10^3 and various less deeply stained bands with an apparent mol. wt range of (14-97) x 10^3.
The influence of culture conditions on the protein patterns was first studied in four strains with different protein patterns. Culture in Nutrient Broth (CM 1, Oxoid), in yeast broth (Lugtenberg *et al.*, 1976), or on Iso-Sensitest Agar (CM 471, Oxoid) had no significant influence on the individual protein patterns. However, when the strains were cultured in an S-2 saline solution enriched with acetate 0.2% w/v (Monod and Wollman, 1947), all produced several extra minor protein bands with apparent mol. wts > 66 x 10^3. The influence of the growth temperature and the age of the culture was also investigated. The protein patterns of strains grown for 10 or 20 h at 30°C or 10 h at 37°C showed no qualitative differences, except for slight differences in the thickness of some minor protein bands (data not shown). In the subsequent experiments, the strains were grown in nutrient broth for 20 h at 30°C. The patterns of the isolates treated in this way were stable and reproducible.
The primary criterion for distinguishing between the patterns was the position of the most deeply stained band with an apparent mol. wt in the range (39.8-46.8) x 10^3. However, the positions and numbers of the other protein bands were also used for discrimination. Patterns were only considered to be indistinguishable if no differences could be observed in any of the protein bands.

Table I. Origin and variety of strains of *A. calcoaceticus* classified on the basis of their cell envelope protein SDS-PAGE pattern. (Prototypes of the patterns are shown in fig. 1).

	Source of strains*			Variety**		
Protein pattern	City	Hospital	Culture collection	glu	haem	Sequential no.
A	R	1 (p)		+	–	1-12
A	R	1 (p)		–	–	13-14
A	R	1 (p)		+	+	15
A	R	2		+	–	16
A	R	3		+	–	17
B1	D	4 (p)		+	–	18-22
B1	D	5 (p)		+	–	23
B2	U	1 (p)		+	–	24-25
B2	N	1		+	–	26
B3	N	1		+	–	27
B4	R	6		+	–	28
C	L	1		+	–	29-30
D1	R	1 (p)		+	–	31-32
D1	R	3		+	–	33
D1			LMD 79.41	+	–	34
D1			B. Vogel	+	–	35
D2	N	1		+	–	36
D3	N	1		+	–	37
E1	L	1		+	+	38
E2	R	1 (p)		+	+	39
E3			Gilardi 2890	+	+	40
E4			LMD 70.9	+	+	41
F	R	7		–	–	42
F	R	6		–	–	43

R, Rotterdam; D, Dordrecht; U, Utrecht; N, Nijmegen; L, Leiden.
1, University Hospital; 2-5, smaller peripheral hospitals; (p) hospitals with epidemic increases of *A. calcoaceticus*.
LMD, Culture collection of Laboratory of Microbiology, Delft.
* All the strains were isolated from clinical material with the exception strain 39, which was cultivated from the inanimate hospital environment.
** Variety based on aerobic acidification of glucose (glu) and on haemolysis (haem).

Patterns of classifable strains

A great heterogeneity of patterns was found in the collection of strains studied. By application of samples of common origin in adjoining slots, several concordant patterns could be identified. For the purpose of this study the patterns were arbitrarily coded with capital letters. Patterns that were similar but not identical were given a capital letter, followed by a number.
A survey of strains classifiable on the basis of their protein patterns is given in table I. Prototypes of these strains are shown in fig. 1. Seventeen of the 34

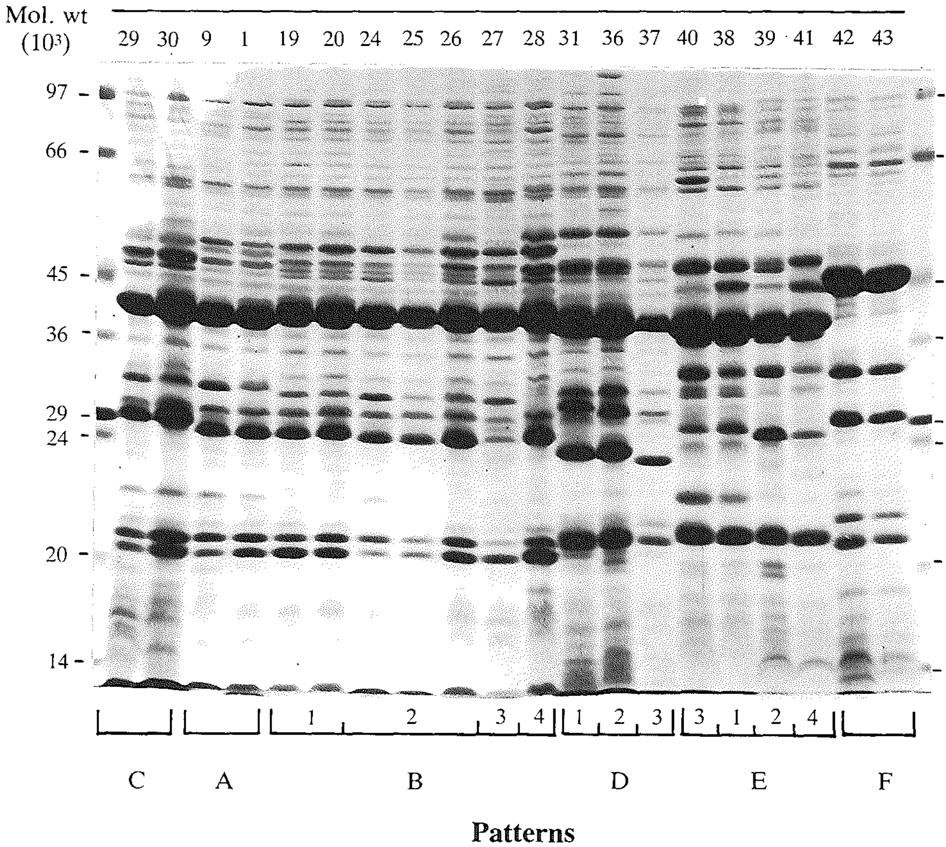

Fig. 1. Cell envelope SDS-PAGE patterns of classified strains of *A. calcoaceticus* (table I). Classification was based on a comparison of all the protein bands. Mol. wt standards are shown on the left. The numbers of the strains correspond to the sequential numbers in table I.

clinical isolates from Rotterdam were classified as having pattern A; 15 of these came from one hospital (University Hospital) and were mostly collected during a period of epidemic increase. The patterns of the six strains from Dordrecht (strains 18-23) were indistinguishable and were designated as pattern B1. Five of these strains had been isolated in a 14-month period in one hospital. Strains 24 and 25 from Utrecht and strain 26 from Nijmegen were indistinguishable. Their pattern was very similar to B1 but was designated B2 because of the presence of a more rapidly migrating protein with an apparent mol. wt of 27.5 x 10^3. The patterns of a strain from Nijmegen

Table II. Origin and variety of strains with unique cell envelope protein SDS-PAGE patterns. (Several patterns are shown in fig. 2)

Source of strains			Variety*			
City	Hospital	Sample	glu	haem	(n)	Sequential no.
R	1	clin	–	–	(3)	44,45,46
R	1	volunt	–	–	(3)	47,48,49
R	1	environ	–	–	(1)	50
R	2	clin	–	–	(1)	51
R	8	clin	–	–	(2)	52,53
L	1	clin	–	–	(1)	54
...	...	environ	–	–	(1)	55
R	1	clin	–	+	(1)	56
L	1	clin	–	+	(1)	57**
R	1	clin	+	–	(1)	58
R	2	clin	+	–	(1)	59
R	8	clin	+	–	(1)	60
L	1	clin	+	–	(2)	61,62
N	1	clin	+	–	(3)	63,64,65
...	...	environ	+	–	(3)	66,67,68
R	1	clin	+	+	(1)	69
L	1	clin	+	+	(1)	70**
Collections						
ATCC 15309			–	–		71
ATCC 9957			–	–		72
LMD 81.109			–	–		73
ATCC 19639			–	–		74
ATCC 23055			+/–***	–		75
ATCC 13809			+	–		76
LMD 82.54			+	–		77
NCTC 7844			+	–		78

Cities and hospitals are as in table I except 8, a children's hospital.
Samples: volunt, from skin of laboratory personnel (strains 47 and 49) and hospital staff (strain 48); environ, from door (strain 50) and soil (strains 55 and 66-68); clin, from patients.
* Variety based on aerobic acidification of glucose (glu) and on haemolysis (haem); (n), number of strains investigated.
** Protein patterns with two migration major proteins close together, apparent mol. wt (41.0-42.6) x 10^3.
*** The culture comprised saccharolytic and non-saccharolytic bacteria with different protein patterns.

and a strain from Rotterdam differed from those of B1 and B2 in respect of some less deeply stained protein bands and were labelled B3 and B4 respectively. The patterns of two strains from Leiden (29 and 30) were indistinguishable from one another but differed from patterns A and B1-4 in

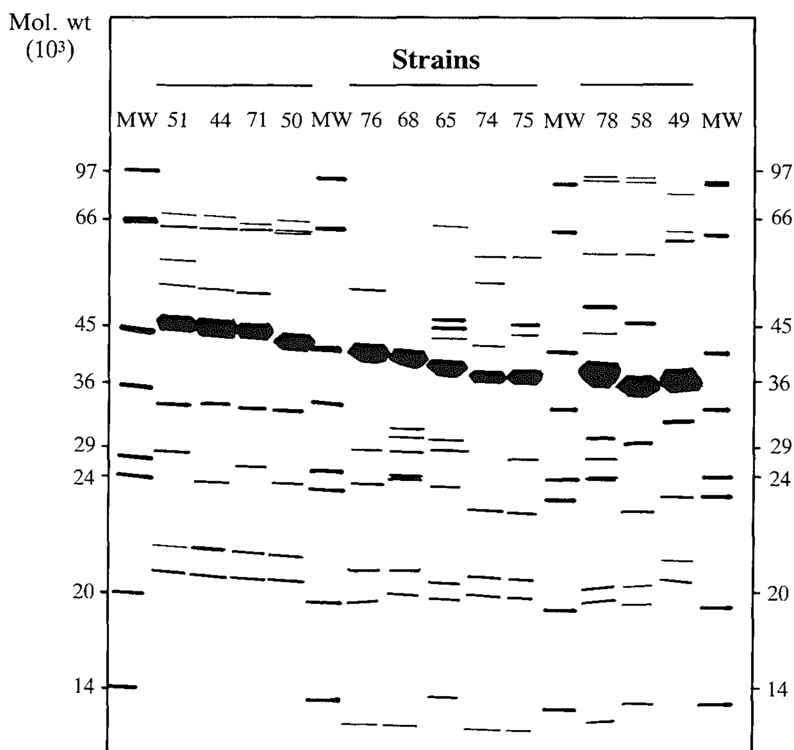

Fig. 2. Cell envelope protein SDS-PAGE patterns of 12 *A. calcoaceticus* strains which could not be classified. Details of the strains are given in table II.
Mol. wt standards are shown on the left and right and between sets of strains (MW).

respect of some minor proteins with mol. wt ranges of (28.2-29.7) x 10^3 and (48.0-54.4) x 10^3. Their patterns were, therefore, classified as pattern C. The patterns classed as D1-3 were similar, but not identical. In these, the most deeply stained protein had an apparent mol. wt of 41.7 x 10^3, and some other characteristic proteins had apparent mol. wts of (25.7-26.3) x 10^3 and (19.1-20.4) x 10^3. Pattern D1 was observed in several strains from Rotterdam; two of these were isolated in one hospital. However, pattern D1 was found not only in strains from a single geographical source but also in some strains from culture collections.

The next group of patterns was found in strains from different sources. The patterns exhibited small differences in minor proteins and were, therefore coded as E1-4. These patterns were characterised by a major protein band (apparent mol. wt 39.8 x 10^3) that was scarcely separated from a thin band of a protein of somewhat higher mobility. Other proteins characteristic of this

group had apparent mol. wts of 20.4 x 10^3 and 34.7 x 10^3.
Two isolates from Rotterdam had indistinguishable patterns (pattern F) characterised by the low mobility of the major protein, corresponding to an apparent mol. wt of 46.8 x 10^3.
Almost all the strains of patterns A, B1-4, C and D1-3 were saccharolytic and non-haemolytic (table I). The strains of patterns E1-4 were all saccharolytic and haemolytic. The two pattern-F strains were non-saccharolytic and non-haemolytic.

Patterns of unclassifiable strains

The patterns of 35 strains were unique and very heterogeneous. The strains in this category (table II) came not only from clinical material but also from the skin of healthy persons, from environmental specimens and from culture collections. The strains varied in saccharolytic and haemolytic properties. Several strains whose most deeply stained protein was of relatively low mobility - apparent mol. wt (44.8-47.2) x 10^3 - were of the asaccharolytic and non-haemolytic variety (e.g., strains 51, 44, 71 and 50; fig. 2). The mobility of the corresponding protein was greater in most of the saccharolytic, non-haemolytic strains (e.g., strains 76, 68, 65, 78 and 58; fig. 2). However, no clear relationship could be established between the mobility of the major protein and saccharolytic capacity.

DISCUSSION

The membrane protein profiles of Gram-negative bacteria can be influenced by culture conditions (Lugtenberg *et al.*, 1976; Brown and Williams, 1985). In introductory experiments aimed at determining reproducibility, variation in the duration and the temperature of incubation and in the composition of some enriched media was found to have little effect. In contrast, several extra minor protein bands were observed when the cells were grown in acetate-mineral medium.
An initial survey revealed a great heterogeneity of electrophoretic patterns in the strains included in the study. When samples were classified on the basis of origin and, to a certain extent, on the basis of saccharolytic activity and haemolysis, it was possible to identify some concordant patterns. Two groups of strains could be distinguished. One group comprised strains whose patterns could be classified (table I). The other group consisted of strains with patterns that were unique and, therefore, unclassifiable (table II). Twenty-three strains in the first group were assigned to patterns A and B1. Each of these patterns

appeared to be associated with a particular geographical location, because the majority of the strains with a common protein pattern had been isolated in one hospital. This pointed to an epidemiological relationship between strains of one pattern and one source. Some SDS-PAGE classification systems for Gram-negatives, e.g., those for *Haemophilus influenzae* type b and *Neisseria meningitidis*, are based on differences in major proteins (Barenkamp *et al.*, 1981; Mocca and Frasch, 1982). Because the principal protein bands of the *Acinetobacter* strains were not sufficiently discriminative, the entire protein profile was used for comparison. In the case of the classified strains, the differences between the patterns within a group (e.g., B1-4) and even between the patterns of groups A, B1-4 and C (fig. 1) were small and confined to minor proteins.

The strains in this study were also differentiated on the basis of saccharolytic activity and haemolysis. The taxonomic significance, however, of the capacity to acidify sugar has been questioned (Henriksen, 1973). Saccharolytic and asaccharolytic strains are assumed not to differ in pathogenicity (Rosenthal, 1978). Nonetheless, nosocomial infections appear to be frequently associated with acinetobacters of the saccharolytic variety (Glew *et al.*, 1977; Ramphal and Kluge, 1979; Holton, 1982). With few exceptions, the classified strains which were assigned to patterns A, B1-4, C and D1-3 were of the saccharolytic variety. On the basis of an extensive study of strains of *Escherichia coli*, Achtman *et al.* (1983) asserted that membrane protein patterns and other parameters such as biotype are not causally related. In their view, protein patterns are more suitable for identifying clonal relationship.

Thirty-five of the 78 strains had unique protein patterns which could not be classified. These strains were of varied geographical and ecological origin and also differed in their saccharolytic and haemolytic properties. The heterogeneity of protein patterns observed suggests that there are many different *Acinetobacter* strains. Other studies have led to the conclusion that *Acinetobacter* is a heterogeneous genus. This followed from the observation that the mol% G + C of the bacterial DNA of *Acinetobacter* strains can vary over a range of 38-47 (Henriksen, 1976). Moreover, it has been found that strains can differ considerably in nutritional properties (Baumann *et al.*, 1968). Recently 12 DNA hybridisation groups (genospecies) have been found among a collection of 85 *Acinetobacter* strains; 28 phenotypic characteristics were found useful for the differentiation of these genospecies (Bouvet and Grimont, 1986).

The purpose of this study was to investigate whether SDS-PAGE protein patterns can be used to classify acinetobacters. In view of the heterogeneity of the patterns that were observed, development of an absolute classification method is not possible at present. Because the patterns were stable and a relationship with particular geographical sources was found for some patterns,

it would, however, appear that the method can be used for the relative classification of strains in well-defined clinical situations.

Though a relative classification of this kind can be useful for nosocomial epidemiology, it remains desirable that a general classification method should be developed. The combined use of different methods, such as DNA hybridisation, SDS-PAGE typing, immunoblotting, serotyping and biotyping, could contribute to the identification of strains of epidemiological or clinical importance.

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CHAPTER 3

TYPING OF *ACINETOBACTER CALCOACETICUS* STRAINS ISOLATED FROM HOSPITAL PATIENTS BY CELL ENVELOPE PROTEIN PROFILES

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SUMMARY

The usefulness of sodium dodecyl sulphate-polyacrylamide gel electrophoresis patterns of cell envelope proteins for classifying strains of *Acinetobacter calcoaceticus* was studied using 129 isolates from 16 in-patients in a teaching hospital. In 11 patients, all of the isolates from each patient exhibited the same pattern irrespective of the body site or time of isolation. The patterns of the isolates from four other patients were indistinguishable, with the exception of one isolate per patient. In the isolates from one patient five patterns were observed. In several cases isolates from different patients exhibited the same pattern. The relative frequency of some of these patterns was low. Epidemiological data were compatible with the assumption that the concurrent presence of bacteria of these patterns in the patients was the result of cross-infection. For one pattern, which was seen in seven patients, cross-infection could not be substantiated. On the basis of analysis of electrophoretic patterns in combination with epidemiological data on a number of strains it is concluded that cell envelope protein profiles appear to be a useful aid in studying the dissemination of *Acinetobacter* in the hospital environment.

INTRODUCTION

In recent years several epidemic outbreaks of *Acinetobacter calcoaceticus* isolations have occurred in hospital departments (Holton, 1982; Stone & Das,

1985) and reliable typing systems are needed in order to be able to study the dissemination of *Acinetobacter* in such situations. Different methods of typing acinetobacters have been described, such as serotyping (Das & Ayliffe, 1984), bacteriocin typing (Andrews, 1986) and the combined use of biochemical tests and phage-typing (Joly-Guillou *et al.* 1984).
The technique of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of membrane proteins has been found to be a useful aid in distinguishing between bacterial strains (Loeb & Smith, 1980; Blaser *et al.* 1983). This method can also be used to establish identity or non-identity of strains of *Acinetobacter* (Alexander *et al.* 1984; Dijkshoorn, Michel & Degener, 1987). Studies of certain Gram-negative bacteria have shown, however, that intra-strain variation of SDS-PAGE patterns can occur. For example, individual strains of *Neisseria gonorrhoeae* can vary in pattern when isolated from different body sites in a patient or after transfer to another host (Zak *et al.* 1984).
In this study the use of SDS-PAGE cell envelope protein patterns as an epidemiological marker was examined. To obtain an impression of the stability or instability of the expression of the proteins, the patterns of multiple isolates from a number of patients were compared. In analysing the patterns both the time of isolation and the location of the patients were taken into account.

MATERIALS AND METHODS

Microorganisms

A total of 129 isolates from 16 in-patients in a 1,000-bed teaching hospital were studied. Clinical isolates were cultivated from samples which were sent to the bacteriological laboratory for clinical reasons at different points of time throughout the hospitalization period. If clinical samples were positive for *Acinetobacter*, epidemiological samples were taken from different body sites of the patient in question within a period of 1-2 weeks, using a standard procedure. Moist swabs were taken from the scalp, the forehead, the external auditory canal, the nose, the throat, the axilla, the palm, the groin, the rectum and the toe web. The swabs were shaken in a liquid medium consisting of an S-2 saline solution (Monod & Wollman, 1947) enriched with 0.2% sodium acetate (w/v). The media were incubated for 24 h at 30°C in a shaking water bath. Subcultures of these media on blood agar and on MacConkey Agar (CM7b, Oxoid) were studied for *Acinetobacter*. The bacteria were identified as *Acinetobacter* by conventional methods (Cowan, 1974) and divided into phenotypic varieties on the basis of the aerobic acidification of glucose and haemolysis on blood agar enriched with 5% sheep blood.

SDS-PAGE of cell envelope proteins

The preparation of the cell envelopes and subsequent SDS-PAGE were carried out essentially as described by Lugtenberg *et al.* (1975), with some minor modifications (Dijkshoorn, Michel & Degener, 1987). Briefly, cultivated cells were disrupted by ultrasonic treatments. Cell envelopes were isolated by differential centrifugation. SDS-PAGE was performed with a Protean Dual 16 cm slab cell apparatus (Bio-Rad, Richmond, California, USA). Discontinuous systems with a stacking and running gel of 3% and 11% acrylamide respectively were used. The gels were run at a constant current of 30 mA for the stacking gel and 35 mA for the running gel in approximately 3.5 h. The proteins were stained with Fast Green FCF (Sigma Chemical Co., St. Louis, MO, USA).
The relative molecular mass (M_r) of the proteins was determined by comparing their electrophoretic mobility with that of the standard proteins phosphorylase B (97 400 (97.4K)), bovine albumin (66K), ovalbumin (45K), glyceraldehyde-3-phosphate dehydrogenase subunit (36K), carbonic anhydrase (29K), trypsinogen (24K), trypsin inhibitor (20.1K) and lactalbumin (14.2K) (Sigma).

Classification of protein patterns

The patterns were classified by visual inspection on the basis of similarity. The primary criterion for distinguishing between the patterns was the most deeply stained protein band with an M_r in the 40-52K range. Other bands, for most patterns in the M_r range of 18-55K, were also evaluated. The patterns were coded with capital letters. Patterns which were similar but not identical were designated by capital letters followed by a number.

RESULTS

Electrophoretic patterns of the isolates

The first study was to determine whether multiple isolates from individual patients exhibited variations in their protein patterns. All the isolates from different patients were then compared and classified. Uniform patterns per patient were found for 11 of the 16 patients (Table 1). The patterns of four isolates from each of patients III, VII and VIII are shown in Fig. 1. The profiles of patients XII-XV were indistinguishable with the exception of one protein pattern (Table 2). Five different patterns were found in the isolates of patient XVI.

Table 1. Patients whose isolates were uniform in protein pattern

Patient	Protein pattern*	Number of samples	Origin of samples** Clinical (*n*)	Epidemiological	Sampling period (range in days)
I	A	8	sp	ea, no, th, ax, hp, gr, an	6
II	A	8	vg, ur	sc, fr, no, ax, hp, an	22
III	A	11	sp (2), td	fr, no, th, ax, hp, gr, an, to	13
IV	A	2	pu	to	10
V	B2	11	ea, lq (7)***	sx, ax, gr	15
VI	G	5	ur (3)	th, gr	27
VII	H	10	um	fr, ea, no, th, ax hp, gr, an, to	9
VIII	D3	7	ct, sp (2)	ax, hp, gr, to	35
IX	J	2	df	th	8
X	K	3	ur	th, an	8
XI	E5	4	wo (3), sp	–	46

* SDS-PAGE protein patterns of cell envelopes are shown in Fig. 2.
** Clinical samples: (*n*), number of multiple samples; sp, sputum; vg, vagina; ur, urine; td, thorax drain; pu, pus; ea, external auditory canal; lq, liquor cerebrospinalis; um, umbilicus; ct, tip of catheter; df, draining fluid; wo, wound; epidemiological samples: sc, scalp; fr, forehead; no, nose; th, throat; ax, axilla; hp, palm of the hand; gr, groin; an, anus; to, toe web.
*** One to three colonies from each sample were separately subcultured and investigated for protein pattern.

Table 2. Patients whose isolates were assigned to different patterns

Patient	Protein pattern*	Number of samples	Origin of samples** Clinical (*n*)	Epidemiological	Sampled on day***
XII	A	2	wo	—	1, 15
	D1	1	—	an	43
XIII	G	6	sp	sc, no, th, gr, to	1, 7
	A	1	—	an	7
XIV	L	7	wf (4), ur, ab	an	1, 8, 10, 17
	O	1	—	sc	8
XV	H	15	um	fr (2), ea (2), no, th, ax, hp (3), gr, an, to (2)	1, 4, 9
	P	1	—	ax	9
XVI	A	1	ur	—	ba
	M	1	wf	—	ba
	B1	10	sp (3)	sc, fr, ea, no, th, an, to	1, 3, 10, 21
	L	11	bl (6), al, dr	ax, hp, gr	ba, 1, 11, 16, 17, 18
	N	1	bl	—	17

* SDS-PAGE protein patterns of cell envelopes are shown in Fig. 2.
** See corresponding notes to Table 1; ab, abdomen; bl, blood; al, arterial line; dr, drain; wf, wound fluid.
*** Samples were obtained on several days. The numbers indicate the day on which samples were obtained; ba, recovered from clinical samples in another hospital before admission.

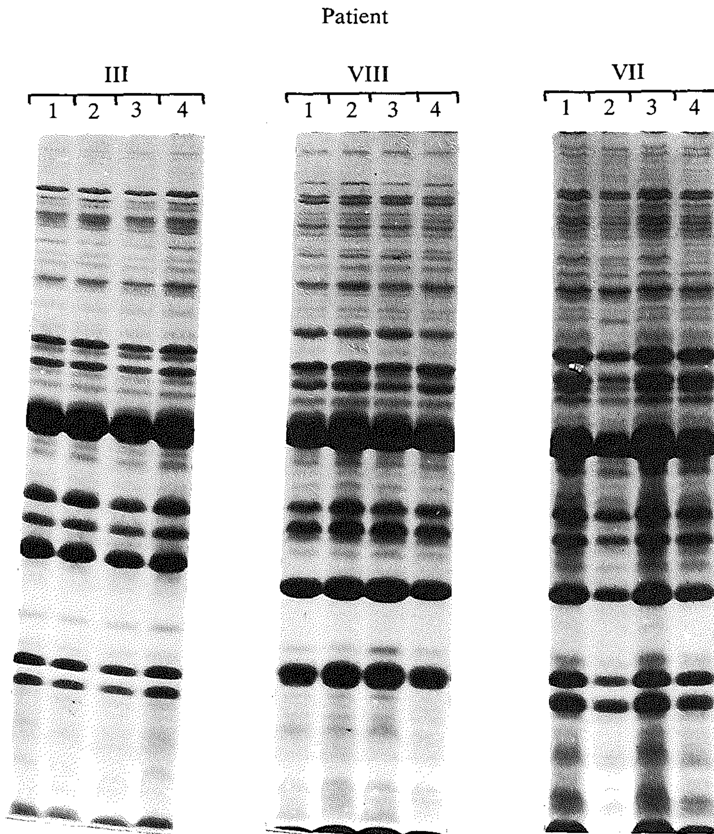

Fig. 1. SDS-PAGE cell envelope protein patterns of multiple isolates of *A. calcoaceticus* from three patients. Patient III: 1, sputum; 2, thorax drain; 3, sputum of a later date; 4, forehead. Patient VIII: 1, axilla; 2, toe web; 3, venous catheter; 4, sputum. Patient VII: 1, umbilicus; 2, forehead; 3, external auditory canal; 4, anus.

The patterns observed in the present study (Fig. 2) were compared with the patterns A, B1-4, C, D1-3, E1-4 and F described in a previous report (Dijkshoorn, Michel & Degener, 1987). In a number of cases, patterns in the present study were found to be concordant with patterns described previously. For example, it can be seen in lanes 5 and 6 of Fig. 2 that the pattern of an isolate from patient III corresponds to pattern A as defined in the earlier study. Similarly, the profiles of lanes 3, 4, 11, 12 and 13 in Fig. 2 could be classified as patterns B2, B1, D3, D1 and E5. Patterns not previously observed were coded with the letters G-P. Patterns M, G, B2, B1, A and L differed from

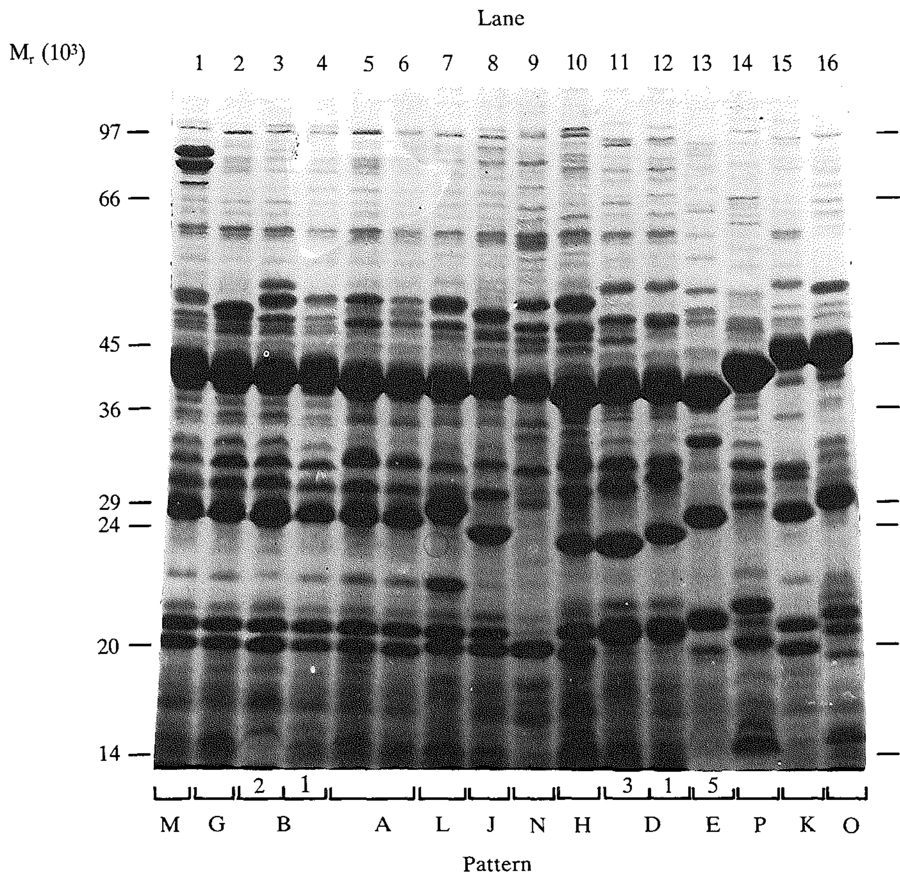

Fig. 2. SDS-PAGE patterns according to which the isolates of *A. calcoaceticus* were classified. The samples were arranged on the gel on the basis of their similarity. The lane 6 isolate was classified in a previous study as pattern type A. The others are described in this study. M_r, relative molecular mass of calibration proteins.

one another in minor protein bands of M_r>45K. In the case of patterns L, J, N, H, D and E5, differences were also observed in minor protein bands of M_r 18-34K. Patterns P, K and O were distinguished by major protein bands with an M_r range of 43-52K and also by various minor protein bands.

Phenotypic varieties

Like the previously isolated acinetobacters with the patterns designated as E1-4, the isolates of pattern E5 acidified glucose and were haemolytic. The

acinetobacters of patterns O and P were asaccharolytic and non-haemolytic. The acinetobacters of the other patterns were saccharolytic and non-haemolytic

Variation and non-variation of protein patterns of isolates from individual patients

The isolates of the patients with uniform patterns, patients I-XI (Table 1), were cultivated from a variety of samples. In most of the patients, after primary isolation from clinical samples the bacteria were also cultivated from various parts of the skin, from the nose and throat and from anal swabs. Positive samples were obtained over a period varying from 6-46 days.
In order to investigate whether the intra-patient variation observed in patients XII-XVI (Table 2) could be connected with the presence of different strains, data on the origin, the protein pattern and other phenotypic characteristics of the isolates was compared. In the case of patient XII acinetobacters of pattern A were isolated, twice from the fluid of a leg wound, at an interval of 15 days. The deviant isolate with pattern D1 was isolated 28 days after the last wound culture from an anal swab. The bacteria with the deviant patterns O and P in patients XIV and XV (Table 2) were isolated from the skin. These isolates deviated from the others not only in protein pattern but also in their inability to acidify glucose.

Identical patterns found in isolates from different patients

The occurrence of common patterns (A, G, H and L) in acinetobacters isolated from different patients (Tables 1 and 2) could be the result of cross-contamination or contamination from common sources. The hospital records were therefore examined to discover whether patients with isolates with the same pattern had been nursed in the same ward in the same period. Four such cases were found. Acinetobacters of pattern A were isolated successively in patients I, II and XII (Fig. 3). The patients had been nursed in several surgical intensive-care units (ICUs). For a short time patients I and II were together in the surgical ICU I. A few days later patient XII spent a day together with patient II in the surgical ICU I. Though cross-infection between the patients may have taken place, this cannot be proved as the relative frequency of pattern A was rather high (7 cases out of 16 patients, Tables 1 and 2).
Acinetobacters of pattern G were cultivated from various samples from patients VI and XIII, who spent 2 weeks together in the surgical ICU II. In patients XIV and XVI, who were some time together in the same ICU,

Fig. 3. Times and places at which *A. calcoaceticus* was isolated from patients. Only one of various concurrent isolates of a given pattern is shown. Patients are indicated by Roman numerals. Further details are given in Tables 1 and 2.

acinetobacters of pattern L were found. The patterns G and L were exclusively observed in these patients and had not been detected in the hospital before. On the basis of epidemiological data and the low frequency of these patterns it is suggested that cross-infection between the patients took place.
Acinetobacters of pattern H were cultivated from the navels of twin brothers, patients VII and XV, immediately after birth (Fig. 3). Later the bacteria were cultivated from epidemiological samples from various skin and mucous membrane surfaces. The twins were probably colonized from a common source immediately after birth.

DISCUSSION

Variation in the expression of cell envelope proteins could be of clinical importance, because it may enable bacteria to escape from immunological surveillance (Sparling, Cannon & So, 1986). If acinetobacters were to display such a variation, the cell envelope protein patterns of the bacteria would not be usable as epidemiological markers. To investigate the variation or non-variation of the protein patterns multiple isolates of *Acinetobacter* from 16 patients were studied. For the majority of patients the isolates were indistinguishable. Some instances of intra-patient variation seemed to be connected with the concurrent presence of different strains (patients XII, XIV and XV). These findings suggest that for strains of *Acinetobacter calcoaceticus* the variability of expression of cell envelope proteins over a limited period is low.
Acinetobacters of the same pattern were isolated from patients who had been nursed in the same unit in the same period. The presence of strains of rare patterns in several patients was suggestive for cross-infection. One pattern (pattern A) was observed in 7 of 16 patients and also in a previous investigation in the same hospital. Although isolation of acinetobacters of this pattern may have been the result of cross-infection, it may also have reflected the general dissemination of the microorganisms in the hospital. Acinetobacters are widespread in nature (Henriksen, 1973). A number of epidemic increases of isolations of *A. calcoaceticus* in hospitals have been described (Holton, 1982; Stone & Das, 1985). For preventive purposes it is important to determine the routes by which the patients acquire the bacteria in these situations and the sites of the body which are colonized. Recently, Allen & Green (1987) reported the isolation of acinetobacters from patients and from the environment during an epidemic. Although for most isolates only the antibiogram was used as an epidemiological marker, it seemed that the bacteria were spread by air.
The patients in our study were sampled for *Acinetobacter* species for epidemiological reasons following primary isolation of the microorganism

from one or more clinical samples. The simultaneous recovery of identical bacteria from various skin and mucous membrane samples from various patients suggests that large areas of the external surfaces may be colonized by acinetobacters for days to weeks. Longitudinal studies of patients are desirable to obtain detailed knowledge of the primary site and the chronological course of colonization by acinetobacters.

On the basis of the foregoing, SDS-PAGE profiles of cell envelope proteins appear to be a useful aid to studying the epidemiology of *A. calcoaceticus*. As patterns of unrelated strains have been found to be heterogeneous (Dijkshoorn, Michel & Degener, 1987), the presence of common patterns in several patients could be indicative for cross-infection or for increased occurrence in the hospital. For the detection of epidemiologically relevant strains in single isolates, however, more needs to be known about the dissemination of particular SDS-PAGE types of acinetobacters both inside and outside the hospital environment.

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CHAPTER 4

CONTROL OF AN EPIDEMIC SPREAD OF A MULTI-RESISTANT STRAIN OF *ACINETOBACTER CALCOACETICUS* IN A HOSPITAL

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ABSTRACT

The spread of a multi-resistant glucose-acidifying *Acinetobacter calcoaceticus* strain in a community hospital was studied. After admission of a colonized patient to the hospital the strain was found in clinical specimens from ICU patients and subsequently from several of these patients after transfer to medical wards. Environmental specimens from the ICU and medical wards were analysed in order to investigate the mode of spread of the strain. Isolates of *A. calcoaceticus* were screened by their antibiotic resistance pattern. In addition, the cell envelope protein electrophoretic profiles were used as epidemiological markers. The multi-resistant acinetobacters all had the same protein profile. To prevent spread of the epidemic strain strict hygienic measures were enforced, e.g. scrupulous cleaning of the room after discharge of any colonized patient and increased attention to the hand hygiene of the medical and nursing staff. Furthermore, the use of antibiotics was restricted. Although this strain was only eradicated with difficulty in the affected patients, it did not spread throughout the hospital. Colonization of patients with the multi-resistant micro-organism was predominantly localized to the ICU.

INTRODUCTION

Bacteria of the genus *Acinetobacter* are aerobic Gram-negative coccobacilli. They have a wide distribution in nature and are readily isolated from water, soil and sewage [17]. All are oxidase-negative and catalase-positive. Although these micro-organisms are of relatively low pathogenicity, they can produce a variety of infections in hospitalized patients [1, 10, 14, 15, 16, 18]. As acinetobacters can be grown from many clinical sources [11] careful judgement is required to decide whether a positive culture represents colonization or actual infection. A high degree of drug resistance may be encountered [8, 12], and this causes problems for the treatment of severe infections. In September 1986 a multiple-trauma patient was admitted to St. Maartens Hospital after some weeks of hospitalization abroad. From the admission report it was known that the patient (the index patient) carried a multi-resistant *Acinetobacter* species strain. Immediately after admission, pus, sputum and bronchial secretions were collected and a multi-resistant glucose-acidifying *Acinetobacter calcoaceticus*, not previously observed in the hospital, was grown. Several measures were taken with respect to hygiene, disinfection and the use of antibiotics in order to avoid cross-contamination and prevent a nosocomial outbreak. The aim of this study was to investigate the extent of spread of the multi-resistant *A. calcoaceticus* strain in the hospital in spite of these measures.

PATIENTS AND METHODS

Bacteriology

The investigation was carried out in a 570 bed community hospital in the period between 19 September and the end of December 1986. Sputum, pus and urine specimens were cultured for *Acinetobacter calcoaceticus* twice a week from patients admitted to the general intensive care unit (ICU) and from other patients at risk. At-risk patients were defined as those receiving ventilation assistance and those being nursed in the same room as patients colonized with the multi-resistant marker strain. Furthermore, environmental samples from bedside cupboards and sinks were taken using swabs, and air samples were obtained by means of a slit-sampler. The samples were inoculated onto blood agar plates which were incubated at 37°C for two days in air.
Identification of Gram-negative isolates was performed on the basis of biochemical characteristics detected by conventional methods including the hydrolysis of Tween 80 [5] and by the API 20NE system. Antibiotic susceptibility was determined by the disc-diffusion technique on Isosensitest agar

(CM 471, Oxoid, Basingstoke, Hampshire, UK). To investigate whether only one strain was involved in the outbreak the cell envelope protein patterns from a collection of 36 *A. calcoaceticus* isolates were compared using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) [6,7].

Prevention measures

Several measures were taken to prevent the spread of the multi-resistant strain. These included scrupulous attention to hand hygiene. The hospital staff were repeatedly instructed to follow an extensive hand-washing procedure, followed by disinfection with 0.5% chlorhexidine in 70% isopropanol, (Hibisol®, ICI, Macclesfield, Cheshire, UK) after each contact with a colonized patient. A clear-cut protocol for hand-washing was placed in an obvious place above each sink in the ICU. Sinks in the ICU and on wards with colonized patients were decontaminated daily by flushing with hot water. The percentage of sinks positive for pathogenic and non-pathogenic micro-organisms on the ICU was clearly lower compared with the wards in which the sinks were not rinsed (not published). If possible, patients underwent source isolation. Immediately after discharge of a colonized ICU patient, the room was scrupulously cleaned. The equipment used, (e.g. the tubes of the ventilation apparatus) was intensively cleaned and sterilized.
Also, restricted use of antimicrobial agents with probably only a moderate effect on the indigenous flora of patients (mainly anaerobic Gram-positive micro-organisms), viz. tobramycin, cefuroxime, metronidazole and co-trimoxazole, was advocated.

RESULTS

Multi-resistant isolates of A. calcoaceticus

A multi-resistant glucose-acidifying *A. calcoaceticus* strain was isolated from a multiple-trauma patient who had been hospitalized in France and treated with antibotics for several weeks. The microorganism was grown from several wounds and from the patient's sputum. The strain was resistant to nitrofurantoin, chloramphenicol, trimethoprim, co-trimoxazole, tetracycline, amoxycillin, amoxycillin-clavulanate, ticarcillin, piperacillin, cefuroxime, cefotaxime, gentamicin and only sensitive to amikacin and tobramycin. This antibiotic resistance pattern had not previously been registered by electronic data processing in the hospital, either for *Acinetobacter species* or for other Gram-negative bacteria. Therefore, this resistance pattern was used to screen

isolates of *Acinetobacter* species. In a later phase of the study the cell envelope protein patterns were used as epidemiological markers.

The first isolation of the multi-resistant *A. calcoaceticus* was observed in September 1986 from the index patient. Subsequent isolates were detected from September 1986 to February 1987. In this period 150 patients were treated in the ICU. The multi-resistant bacteria were isolated from 28 patients, including the index case. These patients were either ICU cases or persons who had been treated in the vicinity of others who had passed through the ICU.

The multi-resistant strain was grown from 12 out of 104 air samples, 13 out of 75 bedside cupboards and from 7 out of 89 sinks in the rooms with patients positive for *A. calcoaceticus* (Table 1). In contrast, a multi-resistant *A. calcoaceticus* was cultivated in only 1 out of 23 air samples from rooms that did not house colonized patients. This positive sample originated from a room adjacent to the one in which a colonized patient was being nursed. The data in Table 1 show that the multi-resistant strain was localized in the ICU and in wards with colonized patients and occurred only rarely in wards without colonized patients ($p<0.01$). Non-resistant acinetobacters were grown from 5 out of 268 samples of wards with colonized patients and from 1 out of 71 samples of wards with non-colonized patients.

The multi resistant strain could not be grown from the fingerprints of five staff members on blood agar plates. However, this was done only once.

Table 1. Multi-resistant *Acinetobacter calcoaceticus* isolated from environmental sources

Source		Isolation		
		Positive	Negative	(n)
ICU and wards with colonized patients	Air	12	92	104
	Bedside cupboards	13	62	75
	Sinks	7	82	89
		32	236	268
Wards with non-colonized patients	Air	1	22	23
	Bedside cupboards	0	27	27
	Sinks	0	21	21
		1	70	71

(n) Total number of specimens studied [$p = 2.99 \times 10^{-3}$ ($p<0.01$), Fisher's test]

SDS-PAGE cell envelope protein patterns

The cell envelope protein patterns of 36 isolates were determined. These isolates were from 24 patients and from 12 environmental samples. Regrettab-

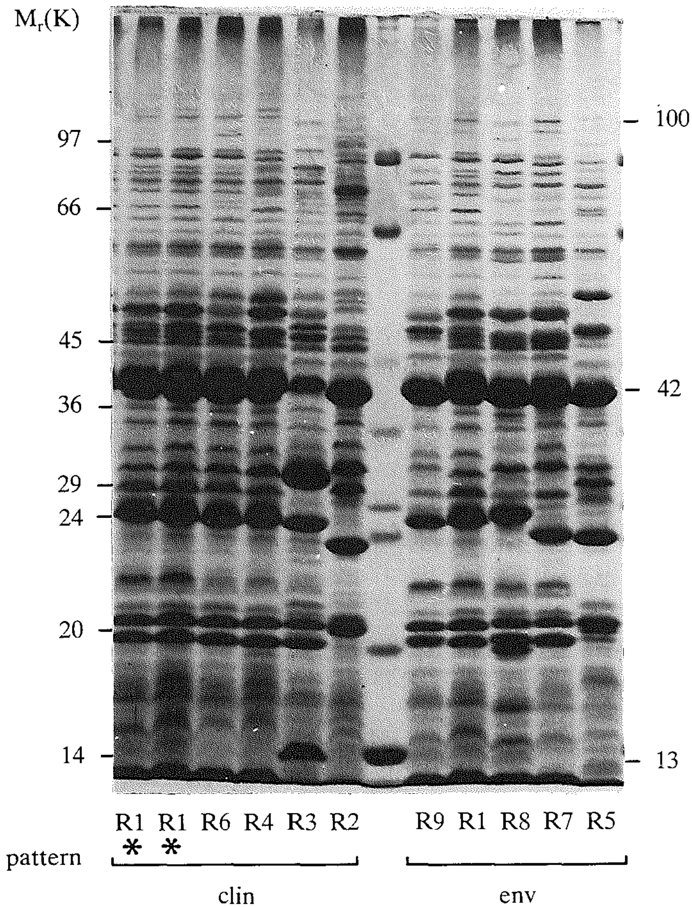

Fig. 1. Cell envelope protein profiles of *A. calcoaceticus*. *Clin*, Clinical isolates from patients; * from different patients; *Env*, environmental isolates; M_r, relative molecular mass of calibration proteins

ly, the original isolates from the index patient were missing from this collection. Nine patterns were identified, which for the purpose of this study, were coded as patterns R1-9 (Fig. 1). Each pattern was characterized by a deeply stained protein band (relative molecular mass, M_r 40 000-42 000 (40-42 K)) and several moderately and weakly stained bands (M_r 13-100 K). One pattern (R1) was seen in the strains obtained from 20 patients (Table 2). These isolates were of the multi-resistant antibiotic spectrum. Seventeen of the 20 patients, positive for pattern R1, were in the ICU when the samples were taken. Of the three other patients, one had been in the unit before; one

Table 2. Cell envelope protein SDS-PAGE profiles of *A. calcoaceticus*

Origin of the isolates	Profile no.	Number of isolates [a]
Clinical		
Sputum	R1	5
Urine	R1	6
Rectum	R1	2
Pus	R1	5
Wound drain	R1	1
Femoral head	R1	1
Urine [b]	R2	1
Post-mortem [b]	R3	1
Pus [b]	R4	1
Urine [c]	R6	1
Environmental		
ICU, non-air	R1	6
ICU, air sample	R5	1
Other wards, sinks	R5, R8, R9	4
Other ward, bedside cupboard	R7	1

[a] Each clinical isolate from a different patient
[b] Samples with strains deviating in hydrolysis of Tween 80, or nitrate reduction and in antibiotic resistance pattern
[c] From another hospital

patient was treated in another hospital in the region and one patient was treated in the surgical unit for a total hip replacement. The patterns of isolates from 3 ICU patients were different (patterns R2, R3 and R4, Fig. 1). These isolates also showed differences in antibiotic resistance patterns, in biochemical features or in both (data not shown). One isolate from a patient in the other hospital mentioned above was also assigned to a different pattern (pattern R6).

Five different patterns were seen (Fig. 1) among the environmental isolates. Pattern R1 was found in 6 strains from rooms containing colonized ICU patients (Table 2). The profiles of 5 isolates from medical wards in which colonized patients were being nursed after discharge from the ICU were heterogeneous and different from pattern R1 (patterns R5, R7-R9, Fig.1, Table 2). These strains differed from those with the R1 pattern not only in the protein profile, but also in the antibiotic resistance pattern.

The protein patterns seen in this investigation were compared with patterns B1, B2, D1-D3 which had been described previously [6] (Fig. 2). It appeared that patterns R1, R4 and R6 were similar but not identical to the B1 and B2 patterns found in *A. calcoaceticus* strains from other hospitals in The Netherlands. Reproducible differences, though hardly visible on photographic repre-

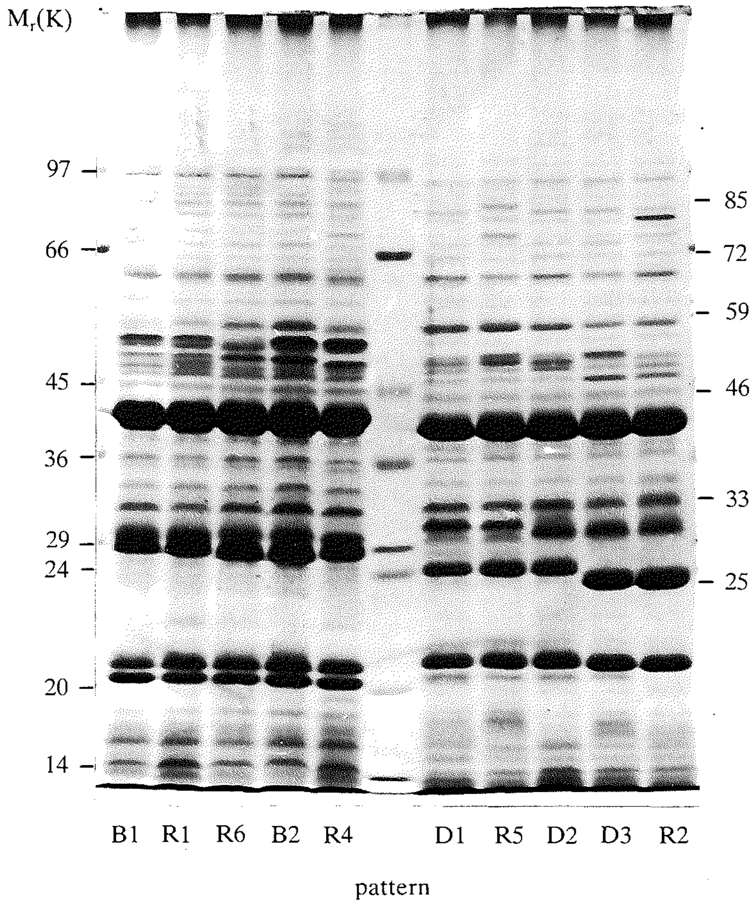

Fig. 2. Comparison of protein profiles of *A. calcoaceticus* of the present study (*R1, R2, R4 and R6*) and of a previous study (*B1, B2, D1-3*) [6]

sentation, were seen in a few minor proteins of M_r range 46-59 K. The patterns R2 and R5 were similar but not identical to the patterns D1-3. Differences were seen in M_r range 25-33 K and 46-85 K.

Multi-resistant isolates with common protein profile

Moderate or heavy growth of the multi-resistant *A. calcoaceticus* strain was cultured repeatedly from 17 out of 28 patients who were colonized (Table 3).

Table 3. Cultural data and protein profile of multi-resistant *A. calcoaceticus* obtained repeatedly in moderate or heavy growth from patients in the intensive care unit

Patient	Isolation from			Stay in ICU before first isolation of the strain (days)	Eradication of the strain during stay in ICU	Protein [a] profile
	Sputum	Urine	Pus			
1 [b]	+	−	+	0	No	n.d.
2	+	+	−	12	No	R1
3	+	+	+	5	No	R1
4	+	+	+	12	No	R1
5	+	−	+	6	No	n.d.
6	+	−	−	1	No	R1
7	+	−	−	14	No	R1
8	+	+	+	19	No	R1
9	+	−	−	7	No	R1
10	−	+	−	5	Yes	n.d.
11	+	−	+	2	No	R1
12	−	+	−	14	Yes	R1
13	−	+	−	7	Yes	R1
14	−	+	−	2	No	R1
15	+	+	+	13	No	R1
16	−	−	+	9	No	n.d.
17	+	+	−	12	No	R1

[a] One isolate from each patient was investigated, n.d., not done
[b] Index patient

The protein profiles of the isolates were determined in 13 of these patients and found to be R1.

This pattern was also seen in 7 other patients from whom the multi-resistant isolate was obtained in small numbers. The bacteria were grown from the respiratory tract (12), from pus (8) and from the urinary tract (10) in the 17 patients shown in Table 3. The time between admission in the ICU and isolation of the multi-resistant *A. calcoaceticus* strain varied greatly (1-19 days, Table 3). The strain was eradicated from the urinary tract of only 3 out of 10 patients. In general, eradication of the strain was only achieved after transfer of the patient from the ICU to a medical ward.

DISCUSSION

Strains of *A. calcoaceticus* are widely distributed in nature [11]. Although these organisms are of low pathogenicity, infections by multi-resistant strains may be life-threatening for patients with altered host defence mechanisms e.g. in cases of surgery, trauma, or endotracheal intubation. Hospital epidemics involving *A. calcoaceticus*, especially among immunocompromised patients, have been reported previously [9, 10, 12, 14].

In this report the epidemic spread of a multi-resistant *A. calcoaceticus* is described. The strain was identified in the first instance by its rare antibiotic resistance pattern. Later, the protein profiles of various isolates were compared. The suspicion that one strain had spread epidemically was corroborated by the finding that the multi-resistant isolates were of one common profile.

Measures were taken in order to prevent a nosocomial outbreak. These included increased attention to the rules of hygiene and restrictions on the use of antibiotics. In spite of these measures the multi-resistant strain was disseminated among the ICU patients. Final eradication of the multi-resistant strain from the ICU area could only be achieved after the discharge of the colonized patients. However, general spread throughout the hospital did not occur (Table 1).

The use of antibiotics favours the selection of resistant bacteria. Restriction of the use of antibiotics, which was daily advocated during the epidemic, has probably been a tool in the prevention of the emergence of multi-resistant acinetobacters throughout the hospital. In environmental samples, the multi-resistant strain was only isolated in the vicinity of colonized patients. Only 1 sample from a ward with non-colonized patients grew a multi-resistant *A. calcoaceticus* (Table 1). This result is in accordance with the study of Allen and Green [1]. These authors observed airborne spread of an epidemic *A. calcoaceticus* strain and also demonstrated that this organism could survive in a dry environment for about a week. The air in the surroundings of a colonized patient (Table 1) may therefore contribute to the environmental reservoir. Reduction of airborne transmission of the bacteria may have been achieved by scrupulous cleaning of the room, including the sinks, bed frames, curtains and ventilation equipment.

The genus *Acinetobacter* is heterogeneous on the basis of physiological characteristics [2]. Deoxyribonucleic acid hybridization (S_1 nuclease method) for identifying the recently described genospecies [3] is not yet available as routine procedure. From the present study it appeared that the SDS-PAGE protein profile can be used to establish the epidemic relationship of the isolates. Minor differences in protein bands seemed to be significant, because these were correlated with differences in antibiotic resistance spectrum and/or biochemical characteristics.

No new multi-resistant isolates of *A. calcoaceticus* were registered after the beginning of February 1987. It seemed that the restricted use of antibiotics, together with the hygienic measures and the specific surveillance for the multi-resistant strain contributed to the eradication of the strain.

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CHAPTER 5

USE OF PROTEIN PROFILES TO IDENTIFY *ACINETOBACTER CALCOACETICUS* IN A RESPIRATORY CARE UNIT

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SUMMARY

The presence of acinetobacters in a respiratory care unit was prospectively studied because of an increase in the number of isolations of *Acinetobacter calcoaceticus*. Cell envelope protein electrophoresis was used to distinguish strains. Eleven protein patterns were observed in isolates from patients and their environment. One pattern (pattern 1) was seen in several patients and environmental samples. Another pattern (pattern 2) was identified repeatedly in samples from skin and mucous membranes of patients in the same ward. After thorough cleaning was undertaken throughout the unit, the pattern 1 strain was no longer cultivated from clinical samples. It is concluded that cell envelope protein electrophoresis is a useful method for tracing of epidemic strains of *A. calcoaceticus*.

INTRODUCTION

Various outbreaks of *Acinetobacter calcoaceticus* in hospitals have been described over the past few years [1-3]. Any increase in the number of isolations of these bacteria in hospital wards should be acted on quickly. Moreover,

a typing method has to be used to determine whether the isolates of an outbreak are identical. Various methods of typing acinetobacters have been described, such as serotyping [4], bacteriocin typing [5], typing based on protein eletrophoretic patterns [6, 7], and biotyping [8].
We used cell envelope protein sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) to investigate an increase in the number of isolations of *A. calcoaceticus* in a respiratory care/ intensive care unit. The analysis of the spread of the bacteria and the origin of the strains were guided by the typing results.

MATERIALS AND METHODS

The study was performed in a respiratory/intensive care unit of a district general hospital with 465 beds. Three beds of the unit are in separate rooms for isolated care; eight other beds are in a large room. This room has an open staff area in its centre and gives open access to a utility room.

Routine measures for hygiene and infection control

Hospital and nursing staff enter the unit through an entrance/dressing room in which clean gowns are put on. Before contact with patients they wash their hands with an ordinary liquid detergent or disinfect them with a solution containing 70% ethanol. At the beginning of the study the unit was cleaned daily with non-disinfectant cleaning agents. Respiratory tubes were thermally disinfected daily in the hospital's central sterilisation department. After prolonged ventilation the respiratory balloon and accessories were sterilised at a temperature of at least 90°C. The moisture exchangers (Servo, Siemens-Elema, Stockholm, Sweden) were fitted with a new sponge every day. Bedpans were cleaned using a bedpan rinser.

Bacteriology

Prospective samples, specifically analysed for *A. calcoaceticus*, were taken in the unit from patients, nursing staff, and from the environment. The patient samples were taken by means of moist swabs from the following parts of the skin and mucous membranes: scalp, forehead, external auditory canal, nose, throat, armpit, palm, groin, toe web and anus. Single swabs from the throat and anus and imprints of three fingers on blood agar and MacConkey agar (CM7b, Oxoid) were obtained from the nursing staff. Samples from environmental surfaces were taken using contact plates with MacConkey agar. Moist

areas were sampled with swabs. Liquid samples were taken from the staff toilet and from buckets of soapy water intended for cleaning the environment. In one isolation room the bacterial contents of the air were investigated using six settle plates filled with blood agar which were exposed to the air for one and a half hours. Samples were taken from four pieces of respiratory apparatus on the ventilator side of the moisture exchanger.
The swabs and samples of cleaning liquids were shaken in an acetate mineral medium [9] for enrichment of acinetobacters. The media were incubated overnight at 30°C in a shaking water bath and subsequently inoculated on to blood agar and MacConkey agar. Acinetobacters were identified using conventional methods [7] and differentiated on the basis of the aerobic acidification of glucose.

SDS-PAGE of cell envelope proteins

The method used has been described in detail elsewhere [7]. Briefly, cell envelope fractions were prepared by ultrasonic disruption of cells followed by differential centrifugation. SDS-PAGE was carried out in a discontinuous system with a stacking and running gel of 3% and 11% acrylamide. The protein profiles were inspected visually for similarity or non-similarity. Both the most densely stained band (of relative molecular mass (Mr) 44 000-47 000) and moderately or weakly stained bands of Mr 18 000-92 000 were examined. Profiles were only considered to be identical if no differences were seen in any of the bands.

RESULTS

Between January and the first week of April 1986 clinical isolates of acinetobacters increased. The bacteria (23 isolates) were cultured from sputum and bronchus secretions from eight of 125 inpatients in the unit and from clinical specimens from seven patients from other wards. Three of these patients had previously been in the intensive care unit for two or three days.

Table 1. Samples for prospective investigation of *A. calcoaceticus* *

	March		April	
	Taken	**Positive**	**Taken**	**Positive**
Patients	168 (16)	18 (7)	61 (17)	25 (10)
Nursing staff	21 (7)	None	None	None
Inanimate environment	207	32	None	None

* Number of patients in parentheses

In March acinetobacters were prospectively found on the skin or mucous membranes from seven of 16 patients in the unit (table 1). Cultures from the throat, rectum, and fingers of the nursing staff were negative. Thirty two of 207 environmental samples were positive for acinetobacter. In April skin and mucous membranes of 10 patients in the unit were positive for *A. calcoaceticus*.

SDS-PAGE patterns

Profiles were classified on the basis of differences of the most densely stained band (Mr 40 000-47 000) and other bands (Mr 18 000-92 000). Eleven patterns were seen in the 23 *Acinetobacter* species isolates obtained from the routine laboratory and in the 75 isolates from prospective samples (fig 1). Table 2 gives the prevalence of the patterns.

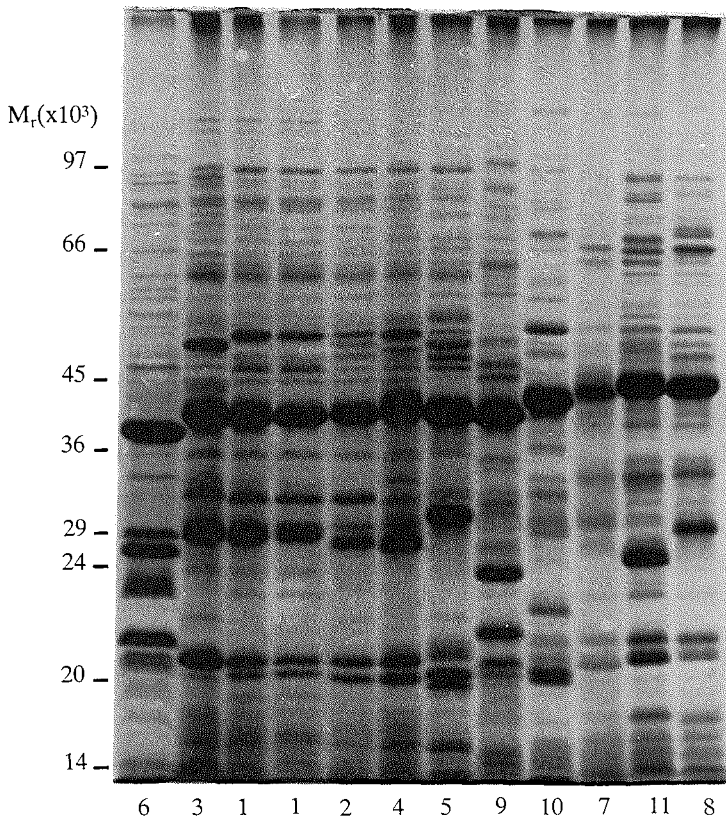


Fig. 1. SDS-PAGE patterns of cell envelope proteins of *A. calcoaceticus* from different patients and from environmental sources. M_r = relative molecular mass of calibration proteins.

Table 2. Prevalence of different protein profiles of acinetobacters

	Prevalence		Environment
Pattern	Patients	Samples	Samples
1	13	35	13
2	11	26	
3,4	1	1	
5			1
6,7,8	1	1	
9			3
10			14
11			1

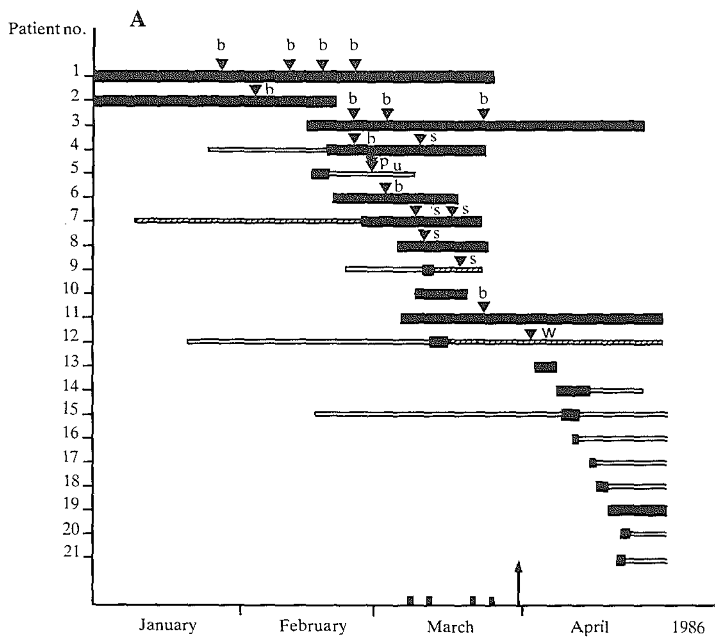
Patterns which were seen only in single isolates or in bacteria from the inanimate environment were not regarded as epidemiologically relevant. On the other hand, pattern 1 was observed in 13 patients and in environmental samples. Pattern 2 was observed in 11 patients. Acinetobacters of pattern 1 and 2 were of the glucose-acidifying variety, with the exception of one isolate.

Samples from patients in the unit

Bacteria of pattern 1 were successively found in clinical samples from 12 patients in the unit (fig 2a). In three patients the bacteria were found only after they had left the unit. Positive samples came from the respiratory tract (n = 16), from urine, pus and wound material (n = 3). In four patients (fig 2b), acinetobacters of pattern 1 were observed in the following 15 prospective specimens from throat (n = 4), forehead (n = 3), nose (n = 2), groin (n = 2), scalp (n = 1), armpit (n = 1), hand (n = 1) and anus (n = 1). In April acinetobacters of a different pattern (pattern 2) were found in 10 patients (fig 2b). These bacteria were either simultaneously or successively cultivated from mouth and anus (11 samples of each), and in nose, armpit, anus and toe web (one sample of each).

Environmental samples

The 207 environmental samples were taken from various sites of the unit - for example from the large nursing room - in the vicinity of infected patients (72 samples), from the utility room (66 samples), from isolation room No II (43 samples), and from respiratory equipment (20 samples). Pattern 1 was



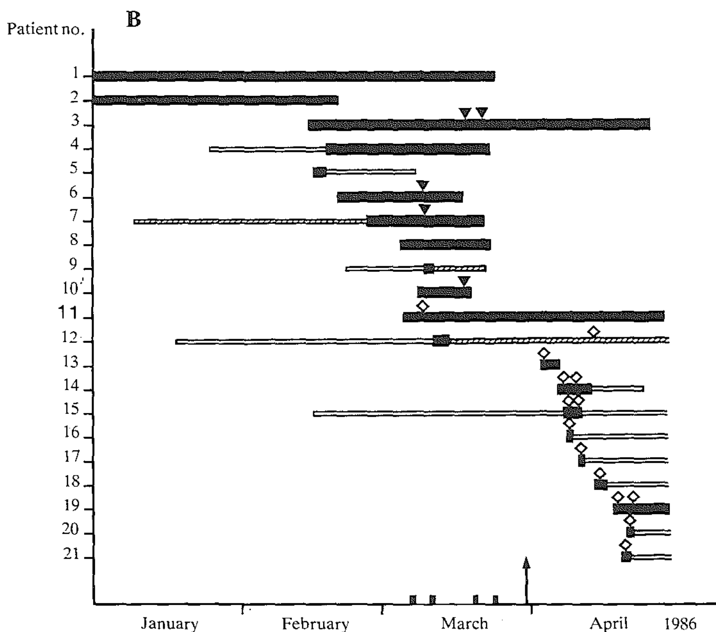


Fig. 2. Isolates of *Acinetobacter* from patients: (a) isolates from samples studied routinely; (b) isolates from prospective samples from skin and mucous membranes. ▼=pattern 1; ◇= pattern 2; b = bronchial secretion; s = sputum; p = pus; w = wound; ▬= ICU; ▨= general surgery; ═= other departments; ▮=sampling of the environment; ↑= disinfection of ICU.

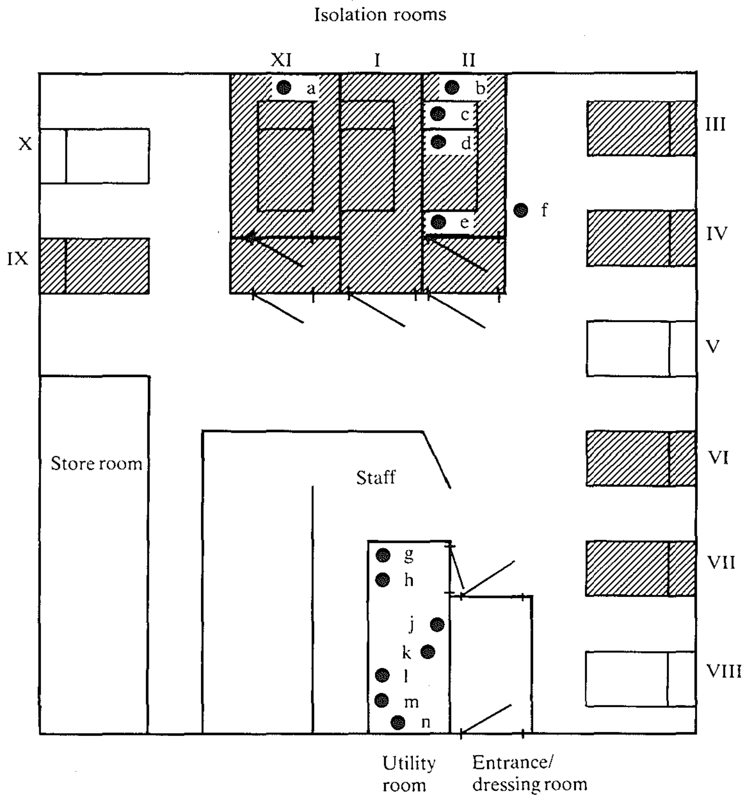


Fig. 3. Sites in the inanimate environment from which acinetobacters of pattern 1 were cultivated. ●=site of isolation; a-d = moisture and external parts of respiratory apparatus; e = cleaning cloth; f = built-in cupboard; g = tuft of fabric; h,j = floor; k,l = draining board; m = bedclothes; n = slop basin.▨ = bed or room with a patient positive for *Acinetobacter*.

observed in 13 of 32 samples from which acinetobacters were recovered. These samples were from both dry and moist sites (fig 3). No acinetobacters were grown on settle plates from isolation room No II, nor were they cultivated from the sample places of the respiratory equipment.

Clinical aspects and intervention measures

The 12 unit patients from whom acinetobacters of pattern 1 were isolated had serious disorders of different kinds. Eleven patients were receiving assisted

ventilation. The time spent in the unit before the first positive acinetobacter culture ranged from one to 60 days, with a mean of 13 days. There were no indications that acquisition of acinetobacters influenced the course of the illness.
Several measures were taken to control the spread of acinetobacters. Compliance with the regulations on hand hygiene was emphasised. In March a switch was made to the use of a phenolic solution for routine cleaning of the environment. By the end of March the unit was systematically disinfected using this solution. No further acinetobacters were isolated from clinical samples.

DISCUSSION

Acinetobacters can cause serious infections in susceptible patients [10] and can become epidemic in hospitals. Environmental spread has been suspected in several reports [1, 3]. Studies of outbreaks have to be supported by typing data of the strains because acinetobacters are ubiquitous.
In this study the increased presence of acinetobacters seemed to be associated with colonisation rather than with clinical infections, but the increase was regarded as a threat to patients and investigated accordingly. On the basis of our findings, contamination of patients from the environment was conceivable. The intensive cleaning and disinfection of the unit and other hygienic measures may have contributed to the disappearance of acinetobacters of pattern 1 from clinical samples by the end of March. By April, samples from skin and mucous membranes of patients were positive for acinetobacters of a different pattern (pattern 2). Although not noticed in clinical samples by routine analysis, the presence of these acinetobacters on the skin and mucous membranes of patients should be a cause of concern.
The genus *Acinetobacter* has been divided into 12 genospecies [11]. The methods for identification of these genospecies and biotyping of genospecies *Acinetobacter baumannii* [12] are not yet widely used. In this study the bacteria were identified as *Acinetobacter calcoaceticus sensu lato*. Both our findings and those from a previous study [9] indicate that SDS-PAGE protein profile typing is a useful method for the relative comparison of strains in small scale epidemiological surveys. Nothing is as yet known about the distribution of patterns in a wide geographic area. The emergence of acinetobacters as a hospital pathogen has created a need for a general classification system. Combined research on strains by means of recent methods such as identification of genospecies [11], biotyping [8, 12] and SDS-PAGE typing could contribute to the development of such a system.

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CHAPTER 6

COMPARISON OF CLINICAL *ACINETOBACTER* STRAINS USING A CARBON SOURCE GROWTH ASSAY

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SUMMARY

A quantitative carbon source growth assay, comprising ten carbon sources, was used to compare *Acinetobacter* strains from three hospitals. The strains had been obtained during episodes of increased prevalence of isolations and were, for each hospital, assumed to be epidemiologically related. This assumption was supported by the electrophoretic protein profiles of the strains. Univariate analysis of growth data showed significant differences between strains from the three hospitals. Moreover, cluster analysis revealed that the major pattern in the data was related to the epidemiological origin of the strains. Exceptions to the epidemic-related pattern were observed. Thus, apart from epidemiological factors, other factors might contribute to carbon source growth profiles of the strains. It is concluded that the carbon growth assay may be useful to distinguish roughly between *Acinetobacter* strains from different sites of origin. Further studies are required to analyse additional factors which influence carbon source growth of strains.

INTRODUCTION

Bacteria of the genus *Acinetobacter* have recently emerged as nosocomial pathogens, which sometimes cause serious infections in hospitalized patients

[1-3]. Therefore, an increase of isolations of these bacteria in hospital wards should be notified quickly. In addition, typing methods have to be used in order to characterize isolates. Several typing methods for acinetobacters have been described, e.g. phage typing [4], bacteriocin typing [5], biotyping [6] and protein electrophoretic typing [7, 8]. These methods are not widely applied. For epidemiological purposes, relatively simple typing methods are needed which can be performed in the routine microbiology laboratory.
The present study was undertaken to investigate whether growth of acinetobacters on various carbon sources could be used to assess similarities between strains. Growth characteristics were determined in a micro-assay by measuring optical densities using a photometer. The method was used to investigate primarily whether *Acinetobacter* isolates from a number of hospitals could be distinguished from their growth patterns. The acinetobacters were obtained from clinical specimens during periods of increased prevalence. Results were interpretated in relation to the epidemiological data of strains and their cell envelope protein profiles obtained by sodium dodecyl sulphate-polyacrylamide gelelectrophoresis (SDS-PAGE).

MATERIALS AND METHODS

Hospitals

The *Acinetobacter* isolates of the study were obtained from three hospitals designated by capitals R, D and V, i.e. the initials of the cities of location, Rotterdam, Dordrecht and Venlo in The Netherlands. Epidemic site R is a 1200 bed teaching hospital. In the period 1981-2, an increased isolation of acinetobacters was observed in several departments of the hospital, in particular in several intensive care units (ICUs). Epidemic sites D and V are district general hospitals with 535 and 570 beds respectively. The increase of isolations in site D was observed in the surgery department in the period May to September 1984. The epidemic spread in site V occurred in the ICU in the period September to December 1986 and has been described in detail elsewhere [9].

Bacteria

Eighty-four *Acinetobacter* clinical isolates from the three epidemic sites R (n=36), D (n=28) and V (n=20) were studied. These strains were obtained from different patients over periods of 9, 4 and 2 months in R, D and V respectively and were selected from those received during the epidemic episodes.

Strains were identified as *A. calcoaceticus sensu lato* [10] by conventional methods [11] and were of the glucose acidifying phenotype.

SDS-PAGE of cell envelope proteins

The method used has been described previously [8]. Briefly, cell envelope fractions were obtained by ultrasonic disruption of cells and fractional centrifugation. For SDS-PAGE, discontinuous systems consisting of a stacking and a running gel of 3% and 11% acrylamide were used. Profiles, consisting of 30-40 bands of different staining intensity in the apparent molecular weight range (M_r) of 14000-97000 (14-97 K), were compared visually.

Carbon source growth assay

The following substrates (C-sources) were used: lactic acid (C1), DL-aspartic acid (C2), D(-)ribose (C3), D(+)xylose (C4), malonic acid (C5), L(-)leucin (C6), L(-)tryptophan (C7), fumaric acid(C8), DL-histidin (C9) and propionic acid (C10). Chemicals C1 (90% pure), C3, C4, C6 and C7 (for biochemical purposes) and C5 and C10 (analytical grade) were obtained from Merck (Darmstadt, FRG), C2 (Sigma grade) from Sigma, St Louis, MO, USA and C9 (>99% pure) from Fluka (Buchs, Switzerland).
The C-sources were added to the basal medium of Gilardi [12] to a final concentration of 0.03M. After pH-adjustment at 6.5, the C-source solutions were stored at -20°C and filter sterilized (Sterivex-GV, Millipore, Bedford, MA, USA) directly before use. Effort was taken to standardize manipulations, growth conditions and incubation times in order to minimize experimental error [13, 14]. Aliquots of 250 μl sterilized saline were applied to each well of the first column of flat bottom microtiterplates (Greiner, Nürtingen, FRG). One of the ten C-sources was added to each of the columns 2 to 11, in aliquots of 250 μl for each well. Fresh bacterial suspensions in saline were prepared from cultures on bloodagar, grown at 30°C for 24 hours. The suspensions were adjusted to an optical density of 0.06 ($\pm$ 0.01), at 623 nm (UC 200 photometer, Vitatron, Meyvis, Bergen op Zoom, The Netherlands), light path 10 mm. Aliquots of 20 μl of the bacterial suspensions were added to the wells of rows A to G. No bacteria were added to the wells of row H in order to check sterility of C-sources. The trays, covered by lids, were incubated at 30°C in a ventilated incubator containing a water reservoir for humidity. After incubation for 48 hr, trays were shaken for 10 seconds (Microshaker, Dynatech, Billinghurst, UK). Optical densities (O.D.) at 620 nm were measured by using a Titertek Multiscan MCC 340 (Flow Laboratories, Irvine, Scotland). The first

column, supplied with saline, served as a blank. For all strains, inoculation of C-sources was performed on one day. All manipulations were carried out by the same two individuals.
In preliminary experiments the reproducibility of the assay was assessed. The intra- and inter-assay variation for the various C-sources is given in Table 1.

Table 1. Coefficients of variation of the assay

C-source	intra-assay	inter-assay
C1	4%	4%
C2	9%	9%
C3	10%	11%
C4	19%	19%
C5	4%	5%
C6	15%	17%
C7	18%	18%
C8	8%	9%
C9	8%	11%
C10	46%	46%

Biometrical analyses

The growth data as determined by the optical densities of the various C-sources were investigated by both univariate and multivariate methods. Univariate comparison between the epidemic sites R, D and V was carried out using the Kruskal-Wallis test (K-W), supplemented by pairwise comparisons using Mann-Whitney's test (M-W) when the former test indicated statistically significant differences ($P<0.05$).
To evaluate the growth data multivariately, cluster analysis was used. By this method the most striking pattern of variation between the strains was revealed. Strains were grouped on the basis of similarities in their growth characteristics. The grouping thus obtained, depicted in a dendrogram, was compared with groupings based on hospital, hospital department, moment of sampling, body sites and other features.
The similarities between the strains, characterized by their growth on C-sources (O.D. x 1000), were calculated using the mean city block distance [15], i.e.

$$d(a,b) = 1/n \sum_{i=1}^{n} | p_a(i)-p_b(i) |$$

where, d(a,b) is the dissimilarity between strain a and b, n is the total number of C-sources (n=10) and $[p_a(i) - p_b(i)]$ is the absolute difference in C-assimilation with the *i*-th C-source between strain a and b.

Clusters were generated by agglomerative cluster analysis. Ward's method [16) was used because of its strong pattern generating capacities [17, 18].

RESULTS

Protein profiles

Protein profiles of strains were inspected for similarity. Strains, the patterns of which were indistinguishable when run in adjoining lanes, were allocated to the same provisional pattern group. Seven pattern groups were established, for the purpose of this study arbitrarily coded with arabic numbers 1-7. The patterns of these groups (Fig. 1) were characterized by one heavily stained band and by 30-40 less densely stained bands.

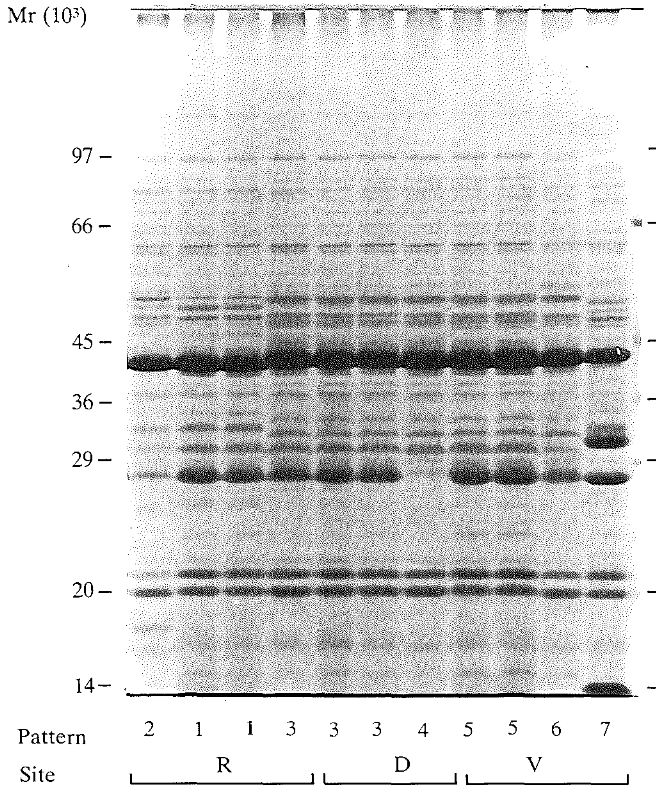

Fig. 1. Different cell envelope patterns observed in *Acinetobacter* strains from sites of origin (hospitals) R, D and V. M_r, apparent molecular weight.

Differences between the patterns were seen in the less densely stained bands, for example, between pattern 1, 2 and 3 in M_r range 45-66K, and between patterns 2, 4 and 7 in M_r range 12-36K. The differences were relatively small, in particular between patterns 3 and 5, but reproducible. For each epidemic site, the majority of strains could be assigned to one specific pattern group, pattern 1 for site R, pattern 3 for site D and pattern 5 for site V (Table 2). For each site, a few strains deviated from the main pattern, e.g. three strains from site R were allocated to pattern 3.

Table 2. Distribution of protein patterns in *Acinetobacter* strains from three epidemic sites*. Patterns are shown in Fig. 1.

epidemic site	pattern 1	2	3	4	5	6	7
R	32	1	3	–	–	–	–
D	–	–	27	1	–	–	–
V	–	–	–	–	18	1	1

* Hospitals in different cities in The Netherlands

Carbon source growth profiles

Table 3 gives the median values of sources C1-C10 according to the site of sampling (i.e. epidemic sites R, D and V). Except for sources C5, C8, C9 and C10, there were statistically significant differences in median levels between epidemic sites. In case of C3 and C7 significant differences were present between all sites. Fig. 2 shows both measurements (C3 and C7), isolated as well as in combination, in relation to the epidemic site. Apart from a few outlying observations, most measurements from site V were well separated from both other sites, while sites D and R were less separated.
The outcome of the cluster analysis of C-growth data of strains using Ward's criterion is shown in the dendrogram (Fig. 3). The major pattern in the data was related to the epidemic sites R, D and V. At a dissimilarity level indicated by the arrow, the following three clusters were distinguished.
Cluster I. This cluster, which showed up as a clearly aberrant group of strains, comprised five strains which grew poorly on one or more C sources. Four strains originated from epidemic site R. One of these strains was aberrant in its quantitative antibiotic resistance pattern (data not shown). One strain from epidemic site V deviated from other strains from the same site with respect to its protein profile (profile 7, Fig.1, Table 2).

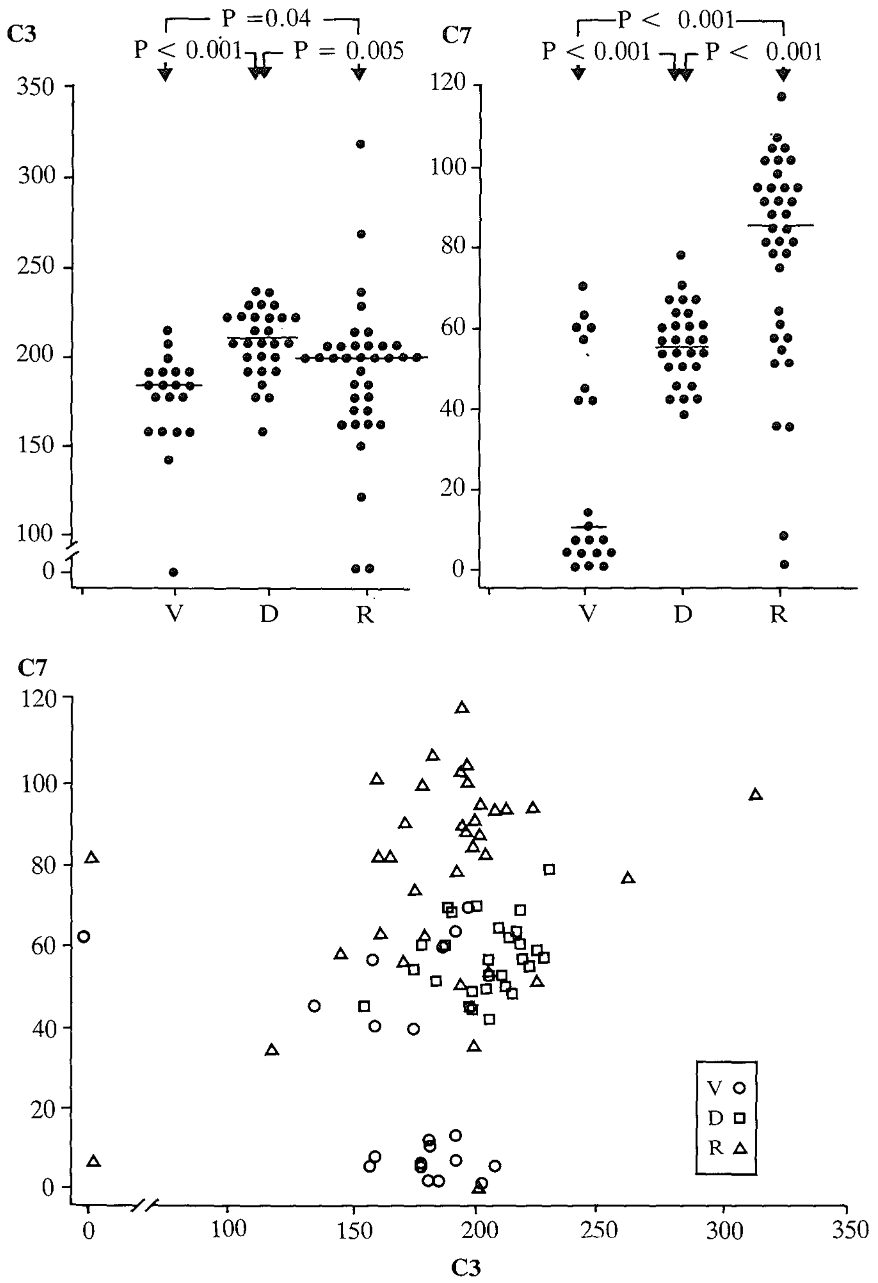

Fig. 2. Measurements of growth on carbon sources C3 and C7 according to sites of origin R, D and V, isolated (upper panel) and in combination (lower panel) expressed in optical densities (x 1000). Bars denote median values.

Table 3. Median values and ranges (between brackets) of measurements of growth on C-sources C1-C10 (optical densities x 1000 at 620nm) according to epidemic site.

	epidemic site			significance (K-W)
	V	D	R	
C1	290 (182-324)	263 (219-285)	262 (3-300)	<0.001
C2	327 (177-342)	328 (289-385)	314 (1-372)	0.04
C3	180 (0-208)	206 (154-230)	194 (1-313)	<0.001
C4	101 (0-130)	123 (87-246)	107 (0-163)	<0.001
C5	351 (0-374)	352 (326-382)	346 (0-388)	0.4
C6	75 (28- 89)	96 (72-124)	101 (38-136)	<0.001
C7	11 (1- 70)	56 (42- 79)	84 (1-119)	<0.001
C8	176 (137-196)	172 (136-200)	170 (4-205)	0.7
C9	239 (208-268)	247 (213-296)	236 (1-268)	0.09
C10	24 (0- 57)	26 (8- 52)	23 (0- 43)	0.7

Statistically significant (M-W: P <0.05) differences:
C1 (V vs D, V vs R), C2 (V vs R, D vs R), C3 (V vs D, V vs R, D vs R),
C4 (V vs D, D vs R), C6 (V vs D, V vs R), C7 (V vs D, V vs R, D vs R)

Cluster II. This cluster, which was well separated from other clusters, contained 12 of a total of 20 strains from site V. The strains were of uniform protein profile (pattern 5) and were associated with a hospital outbreak which was described previously [9].
Cluster III. This contained nearly all strains from site R and all strains from D. Within this cluster, several subgroups were distinguishable. One of these subgroups (subcluster a, Fig. 3) contained exclusively strains from site R; these strains belonged to protein profile group 1 (Fig. 1). Another subcluster (subcluster b, Fig. 3) comprised almost exclusively strains from site D; these belonged to protein profile group 3. Several other subclusters within cluster III were mixtures of strains from D, R and V. Some of the strains in these mixtures deviated from the majority of strains from the same site with respect to their protein profile. For example, one strain in a mixed cluster (subcluster c), originating from site D, was of the deviating pattern 4 (top of the dendrogram); another strain, originating from site V was of pattern 6 (centre of dendrogram), whereas other strains from V were of pattern 5. Also, several strains from site R, which clustered together with strains from D, belonged to pattern 3, i.e. the main pattern for strains from D (subcluster c, upper part of dendrogram).
The strains from site R were obtained from 36 patients in 12 different wards. Twenty-six of the patients had been nursed in more than one ward during the period of hospitalization. No clear relationship was observed between the grouping of these strains within cluster III and department and moment of isolation or other features, e.g. antibiotic sensitivity spectrum.

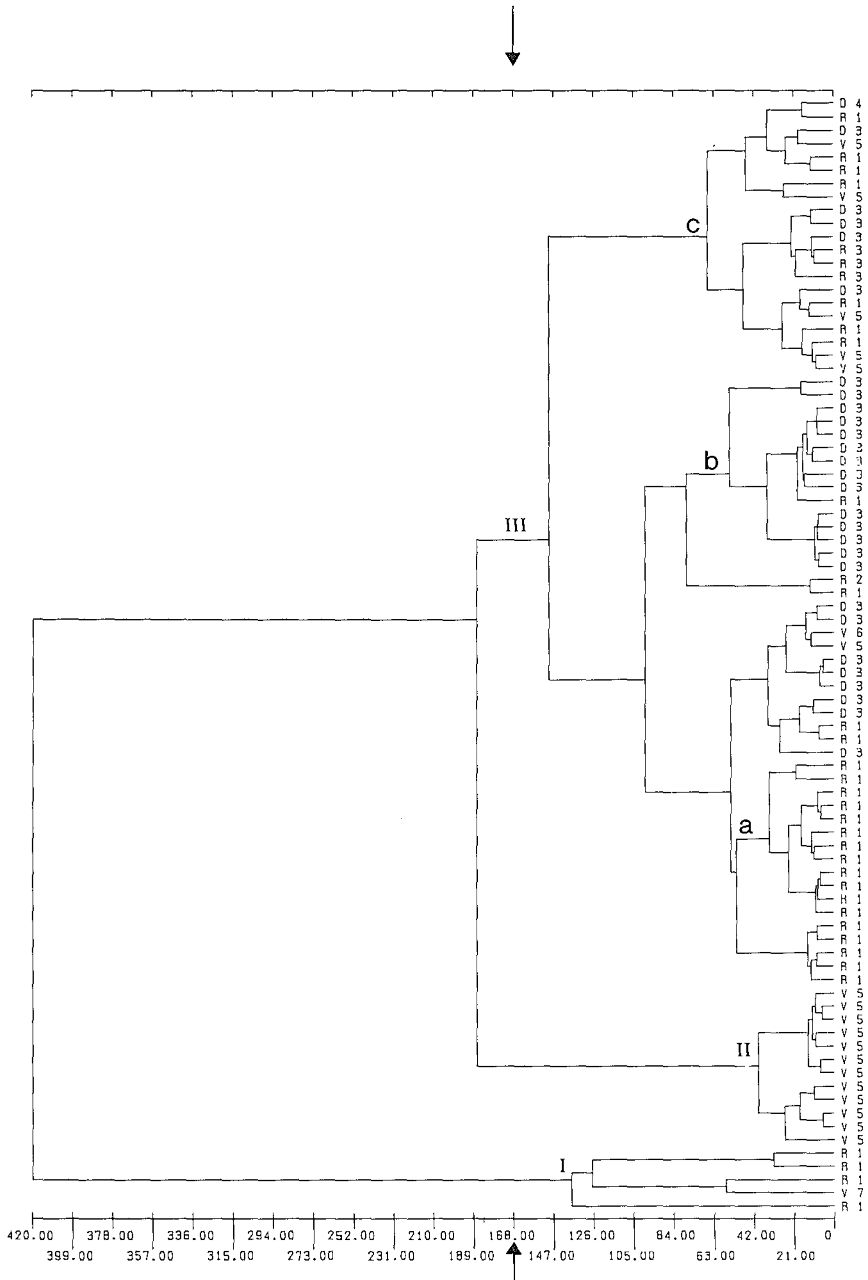

Fig. 3. Dendrogram of cluster-analysis of 84 *Acinetobacter* strains characterized by their growth on carbon sources. Horizontal axis: dissimilarity level at which the strains are grouped. This level is determined by Ward's criterion and based on the mean city block distance. Arrow denotes the cutting level for separation main clusters.
Vertical axis: the strains grouped according to the similarity of their growth on the carbon sources. R, D and V denote epidemic sites of origin (hospital) of the strains. Protein profiles are represented by numericals 1-7; main clusters by I-III and subclusters by a-c.

In addition to the cluster analysis based on Ward's criterion, an analysis was performed using the UPGMA clustering criterion [19]. Results of this second analysis (not shown) largely agreed with the dendrogram shown in Figure 3.

DISCUSSION

The ability of micro-organisms to grow on specific carbon sources is determined by several factors, e.g. the metabolic constitution and cell membrane characteristics. Diversity with respect to the use of different C-sources by bacteria has appeared useful in classification [14, 20, 21]. Baumann and co-workers [22] distinguished nutritional groups within the genus *Acinetobacter*, although the groups were not sharply distinguishable. Recently, Bouvet and Grimont [6, 23] developed schemes for identification and typing of *Acinetobacter* strains, which are largely based on C-growth tests. In contrast to these investigations, the present study has no taxonomic pretention. The purpose of this study was to investigate whether C-growth may be useful in distinguishing between strains, primarily in relation to their epidemiological origin. The number of C-sources used was relatively low. Instead of using qualitative (binary) data, as was done in other studies [22, 23], we used quantitative data. Strains were, for each hospital, assumed to be epidemiologically related. This assumption was supported by the protein profiles of strains. Univariate statistical analysis showed significant differences in median levels between the strains when these were *a priori* separated according to their epidemiological origin. The overlap of the measurements when considered for each carbon source separately, however, was generally large. For some C-sources, differences could only be demonstrated between two of the three hospitals, while other C-sources did not show significant differences. For two C-sources, C3 (D(-)ribose) and C7 (L(-)tryptophan), statistically significant differences existed between all of the three epidemic sites. For C4 (D(+)xylose) and C6 (L(-)leucin), significant differences between some sites were observed, in spite of the moderate reproducibility of results of these C-sources (Table 1). The lack of reproducibility of C10 (propionic acid), possibly caused by the poor growth of the strains on this C-source, might account for the lack of statistically significant differences. In future experiments, this C-source might be replaced by a more discriminating C-source.

By cluster analysis similarities between individual strains and grouping of strains are studied without any *a priori* separation. Thus, the main pattern in the data is studied. This pattern was evaluated for its correlation with several characteristics of the individual strains. The major pattern of the cluster analysis of C-growth data was related to epidemic sites (e.g. cluster II). Exceptions to the pattern were evaluated. Some of these might be explained on the basis of characteristics of the strains. Several strains deviated from strains

from the same site in carbon source growth capacities (strains of cluster I) or in protein profile (e.g. strains of pattern 3 from site R). These strains may have represented occasional clinical isolates, unrelated to the epidemic spread. Strains from site R, a large teaching hospital, were in comparison to those from site V more scattered over the dendrogram. As indicated in the methods section, the period of increased numbers of isolations of acinetotobacters in site R was longer as compared to sites V and D and many departments were involved. Therefore, strains from site R may rather have been of endemic than of epidemic origin. The passage of strains in many patients in a prolonged period may have contributed to the heterogeneity observed in the growth tests. In addition, experimental intra-strain variation [14, 24] may have contributed to the variation, although rigid standardization was applied in the present assay in order to minimize experimental error.
The present study was initiated to develop an easy-to-perform assay for the screening of strains in epidemiological surveys. The cluster analysis revealed a pattern related to epidemic sites. From this, it is concluded that the assay is a useful method in order to distinguish roughly between epidemic sites. However, not all strains assumed to be epidemiologically related were grouped together. Additional factors, largely unknown, contributed to the variation. As this variation may complicate application of C-growth tests in epidemiology, further studies are required to assess these sources of variation.

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CHAPTER 7

NUMERICAL ANALYSIS OF CELL ENVELOPE PROTEIN PROFILES OF *ACINETOBACTER* STRAINS CLASSIFIED BY DNA-DNA HYBRIDIZATION

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SUMMARY

Cell envelope protein profiles, obtained by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE), of *Acinetobacter* strains were compared with DNA hybridization data of the strains. The collection of strains comprised 98 field strains mostly from clinical sources in Sweden and The Netherlands and 22 strains from culture collections. Thirteen DNA groups were represented. Protein profiles were densitometrically recorded and subjected to numerical analysis. When the complete profile was used for analysis, the heavily stained bands outweighed the less heavily stained bands and the clustering did not correlate well with DNA groups. When the analysis was restricted to the upper part of the profile, consisting of minor bands of relatively high molecular weight, most strains belonging to the same DNA group clustered together. Thus, this part of the profile may help to identify strains at the DNA group level, while the complete profile can be used to differentiate strains within DNA groups.

INTRODUCTION

Acinetobacters are aerobic gramnegative coccobacilli which are widespread in nature. Recently, the bacteria have emerged as nosocomial pathogens (Bergogne-Bérézin *et al.*, 1987). In the absence of sufficient criteria for the establishment of different species it was proposed to consider only one species in the genus, *Acinetobacter calcoaceticus* (Henriksen, 1973; Lautrop, 1974). The first DNA-DNA hybridization study of *Acinetobacter* was performed by Johnson *et al.* (1970) who found that the genus contained at least six DNA-relatedness groups. In 1986, Bouvet and Grimont characterized 12 DNA groups ("genospecies") among 85 *Acinetobacter* strains. They proposed four new species - *A. baumannii*, *A. haemolyticus*, *A. johnsonii*, and *A. junii* - and the descriptions of *A. calcoaceticus* and *A. lwoffii* were emended. Yet another species, *A. radioresistens*, was described in 1988 by Nishimura *et al.* In a DNA-DNA hybridization study by Tjernberg and Ursing (1989) 168 *Acinetobacter* strains were investigated and most strains could be identified as members of the DNA groups 1-8 and 10-12 of Bouvet and Grimont (1986). Three new DNA groups were found and given numbers 13-15. The type strain of *A. radioresistens* was shown to be a member of DNA group 12. Just before the submission of this manuscript, a paper of Bouvet and Jeanjean (1989) was brought to our attention, in which five DNA groups of proteolytic acineto-bacters were described (DNA groups 13-17). DNA group 13 of Bouvet and Jeanjean (1989) apparently corresponds to DNA group 14 of Tjernberg and Ursing (1989), whereas no correlation was found for the other DNA groups. Apart from these taxonomic investigations, several methods were developed to differentiate *Acinetobacter* strains for epidemiological purposes, for example, phage typing (Vieu *et al.*, 1979), biotyping (Bouvet and Grimont, 1987), and typing on the basis of protein profiles obtained by sodium dodecyl-sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Dijkshoorn *et al.*, 1987).

In the present study 120 *Acinetobacter* strains, identified at DNA group level, were investigated for their cell envelope protein patterns. SDS-PAGE profiles were recorded by densitometry and subjected to cluster analysis (Pot *et al.*, 1989; Vandamme *et al.*, 1989). Using the complete protein profile, the numerical analysis correlated poorly with the DNA groups; however, a better correlation was found using a restricted part of the protein profile.

MATERIALS AND METHODS

Bacterial strains

One hundred and twenty strains were studied (Table 1). The majority of the collection consisted of field strains, mostly from clinical sources in Sweden and in The Netherlands. The type and reference strains were obtained from the culture collections indicated by their designations, except for strain no. 59, Gilardi 2890, which was obtained from Dr. A.S. Lampe (Leiden). Most strains have been described in earlier publications (Dijkshoorn *et al.*, 1987; Tjernberg and Ursing, 1989); strains no. 107-109 and 111-113 (Table 1) are isolates from The Netherlands and have not been described earlier. The strains, which were studied for their protein pattern, have previously been assigned to provisional groups (Dijkshoorn *et al.*, 1987); the designation of these groups is given in Table 1. Only strains belonging to the described DNA groups 1-8 and 10-14 (Bouvet and Grimont, 1986; Tjernberg and Ursing, 1989) were included. The number of strains in the groups largely reflected the availability of field strains.

DNA relatedness

The strains had been assigned to existing DNA groups using the hydroxy-apatite (HA) method of Brenner *et al.* (1969) as modified by Lind and Ursing (1986) or the quantititative bacterial dot filter (F) method recently described by Tjernberg *et al.* (1989). A few strains were investigated by both methods. Reference DNA was labeled with ^{125}I according to Selin *et al.* (1983). The parameter used was $\Delta\ T_{m(e)}$ (difference in thermal denaturation midpoint between homologous and heterologous duplexes). This parameter could be estimated with both methods. The same reference strains were used as previously (Tjernberg and Ursing, 1989). The criteria for including a strain in a given DNA group were discussed in detail in a previous communication (Tjernberg and Ursing, 1989). The intragroup values for $\Delta\ T_{m(e)}$ ranged from 0.0-5.4°C by the HA method and from 0.0-3.0°C by the F method.

SDS-PAGE of cell envelope protein

Preparation of cell envelopes and SDS-PAGE were performed as described elsewhere (Dijkshoorn *et al.*, 1987) with slight modifications. Briefly, cell envelopes were obtained by ultrasonic disruption of cells followed by fractionated centrifugation. SDS-PAGE was performed using a discontinuous

Table 1. *Acinetobacter* strains of the study arranged according to DNA groups.

Strain: serial no.	Strain: other designation[a]	Specimen[b]	$\Delta T_{m(e)}$ vs reference[c]: F	$\Delta T_{m(e)}$ vs reference[c]: HA	Protein profile[d]
DNA group 1 (A. calcoaceticus)					
1	ATCC23055[T,e]	soil			
2	LMD 22.17(=74[d])	soil	0.0		un
3	67[d]	soil	1.0		un
4	68[d]	soil	1.5		un
5	64[d]	I.V. cath.	1.5		un
6	66[d]	soil	1.9		un
7	42[e]	wound		2.8	
8	132[e]	wound		4.0	
DNA group 2 (A. baumannii)					
9	91[e]	sputum			
10	13[d]	wound	0.0		A
11	10[d]	wound	0.0		A
12	8[d]	toe web	0.1		A
13	26[d]	bronchus	0.2		B2
14	LMD 82.54 (=77[d])	not known	0.2		un
15	NCTC 7844 (=78[d])	not known	0.5		un
16	17[d]	urine	0.5		A
17	11[d]	wound	0.6		A
18	12[d]	urine	0.6		A
19	29[d]	pus	0.6		C
20	4[d]	sputum	0.7		A
21	144[e]	wound	0.2	0.7	
22	20[d]	urine	0.8		B1
23	2[d]	wound	0.9		A
24	18[d]	urine	0.9		B1
25	ATCC 17904[e]	urine	0.6	0.9	
26	27[d]	wound	1.0		B3
27	21[d]	sputum	1.1		B1
28	23[d]	urine	1.1		B1
29	25[d]	sputum	1.1		B2
30	16[d]	urine	1.3		A
31	9[d]	urine	1.3		A
32	22[d]	blood	1.4		B1
33	19[d]	urine	1.5		B1
34	6[d]	urine	1.6		A
35	CCUG 19096[T,e] (=ATCC 19606[T])	urine		1.7	
36	60[d]	sputum	1.8		un
37	3[d]	blood	1.9		A
38	1[d]	sputum	1.9		A
39	7[d]	sputum	2.2		A
40	5[d]	sputum	2.2		A
41	24[d]	I.V. cath.	2.7		B2
42	30[d]	ear	3.0		C
DNA group 3					
43	102[e]	wound			
44	37[d]	drain	0.0		D3
45	31[d]	toe web	0.6		D1
46	162[e]	wound		0.7	
47	36[d]	bronchus	1.6		D2
48	61[d]	sputum	1.9		un
49	63[d]	bronchus	2.0		un
50	32[d]	blood	2.2		D1
51	35[d]	not known	2.8		D1
52	33[d]	urine	2.8		D1
53	34[d] (=LMD 79.41)	not known	2.9		D1
54	59[d]	urine	2.9		un
55	ATCC 19004[e]	CSF	2.2	3.3	
DNA group 4 (A. haemolyticus)					
56	197[e]	wound			
57	38[d]	pus	0.0	0.6	E1
58	LMD 70.9 (=41[d])	not known	0.0	1.1	E4
59	Gilardi 2890 (=40[d])	not known	0.5	1.4	E3
60	ATCC 17906[T,e]	sputum		1.1	
61	39[d]	air	1.4	1.2	E2
62	61[e]	not known		2.1	
DNA group 5 (A. junii)					
63	ATCC 17908[T,e]	urine			
64	53[d]	blood	0.0		un
65	46[d]	not known	0.4		un
66	124[e]	surg. gown		0.8	
67	178[e]	water		3.2	
DNA group 6					
68	ATCC 17979[e]	throat			
DNA group 7 (A. johnsonii)					
69	ATCC 17909[T,e]	gut			
70	92[e]	urine		2.0	
71	68[e]	urine		2.1	
72	97[e]	urine		3.1	
73	112[e]	urine		3.4	
74	134[e]	urine		3.6	
75	153[e]	faeces		4.1	
76	137[e]	urine		4.3	
DNA group 8 (A. lwoffii)					
77	NCTC 5866[T,e]	not known			
78	51[d]	not known	0.0		un
79	45[d]	blood	0.0		un
80	44[e]	prost. secr.		0.0	
81	43[d]	eye	1.9	1.8	F
82	54[d]	not known	2.0		un
83	48[d]	skin	2.2		un
84	49[d]	skin	2.3		un
85	47[d]	skin	2.3		un
86	42[d]	urine	2.4	2.3	F
87	50[d]	door	2.7		un
88	44[d]	wound	2.7		un
89	202[e]	urine		3.5	
DNA group 10					
90	198[e]	urine			
91	113:2[e]	wound		1.0	
92	ATCC 17924[e]	not known		1.1	
DNA group 11					
93	174[e]	contact lens			
94	CIP 63.46[e] (=ATCC 11171)	not known		2.6	
95	LMD 81.109 (=73[d])	not known		3.4	un
96	51[e]	urine		3.6	
97	210[e]	wound		3.7	
98	58b[e]	wound		4.2	
DNA group 12 (A. radioresistens)					
99	109[e]	urine			
100	SEIP 12.81[e]	urine		0.0	
101	152[e]	wound		0.8	
102	26[e]	sputum		1.8	
103	FO-1[T,e] (=IAM 13186[T])	cotton		1.9	
104	73[e]	wound		2.5	
105	50[e]	urine		5.4	
DNA group 13					
106	ATCC 17903[e]	not known			
107	Ac 2624	skin front	0.0		
108	Ac 2285	bronchus	0.4		
109	Ac 2376	sputum	0.4		
110	65[d]	urine	0.5		un
111	Ac 2041	post mortem	0.8		
112	Ac 2284	bronchus	0.9		
113	Ac 2627	rectum	0.9		
114	62[d]	blood	1.4		un
115	100[e]	gastr. fist.		1.4	
116	165[e]	urine		2.0	
DNA group 14					
117	71[e]	conj. secr.			
118	114[e]	CSF		0.6	
119	101[e]	conj. secr.		3.1	
120	ATCC 17905[e]	conj. secr.		4.4	

[a] ATCC, American Type Culture Collection, Rockville, Md, USA; LMD, Culture Collection, Laboratorium voor Microbiologie, Delft, Nederland; NCTC, National Collection of Type Cultures, London, United Kingdom; CCUG, Culture Collection, University of Göteborg, Göteborg, Sweden; CIP, Collection de l'Institut Pasteur, Paris, France; SEIP, Service des Entérobactéries de l'Institut Pasteur, Paris, France; IAM, Institute of Applied Microbiology, The University of Tokyo, Tokyo, Japan; [T], type strain.

[b] All specimens were of human origin, unless otherwise indicated. I.V., intravenous; CSF, cerebrospinal fluid.

[c] $\Delta T_{m(e)}$, difference in thermal denaturation midpoint between homologous and heterologous DNA-DNA duplexes. The first strain of each DNA group was the reference used. F, filter method; HA, hydroxyapatite method.

[d] *Dijkshoorn* et al., 1987; A-F, provisional profile group; un, unique, not classifiable profile.

[e] *Tjernberg* and *Ursing*, 1989.

system with a stacking and a running gel of 3% and 11% acrylamide, respectively. Effort was taken to standardize polymerization times and electrophoresis conditions in order to reduce inter-gel variation. The buffer of the upper chamber of the Protean II cell (Bio-Rad, Richmond, CA, USA) was prepared freshly, two hours before each run. For cooling, the lower buffer chamber was filled completely with buffer, while tapwater circulated through the cooling core. A 20 wells comb was used and routinely 35 μl of a sample, containing 20 μg protein were applied per slot. Gels were run at 20 mA constant current until the front was 1 cm above the bottom of the gel. In each gel, five traces of molecular weight markers were used, which comprised phosphorylase b (97,400 (97.4 K)), bovine albumin (66.0 K), ovalbumin (45.0 K), glyceraldehyde-3-phosphate dehydrogenase subunit (36.0 K), carbonic anhydrase (29.0 K), trypsin inhibitor (20.1 K), lactalbumin (14.2 K), Sigma, St Louis, MO, USA). These traces were evenly distributed over the gel. Gels were stained overnight in 0.1% (w/v) Fast Green FCF (Sigma) in methanol/acetic acid (50/10, v/v) and destained in methanol/acetic acid (45/10, v/v) to a final staining level corresponding to 0.002% (w/v) Fast Green. Gels were dried using the Bio-Rad model 224 slab dryer.

Densitometry and cluster-analysis

Protein electrophoretic patterns were scanned using a model 2202 Ultroscan laser densitometer (LKB, Bromma, Sweden) and a modified LKB Apple Pascal program (GELSCAN) run on an Apple IIe microcomputer equipped with a Transwarp accelerator card (Applied Engineering, Carrollton, TEX, USA). Raw digitized data were normalized and interpolated using the Pascal INTFILE program (B. Pot and P. Casteleyn, unpublished data). Objective comparison and normalization of lanes on different slab gels was achieved by using the five traces of molecular weight markers in each gel. In addition to the starting point and the endpoint of the electrophoretic trace, three protein bands from the molecular weight markers (molecular weight 97.4, 29.0 and 20.1 K) were localized by the INTFILE program. Small individual misalignments of the raw digitized data, collected from different gels or different lanes within one gel were detected by comparison with a standard molecular weight curve, calculated from the mean positions of the molecular weight marker protein bands for all gels analysed. For each gel, the INTFILE program corrected for displacements and reduced the 1,000 points of the raw digitized curve to a 400-point curve by using the Lagrange interpolation algorithm. The resulting 400-point traces, with all necessary descriptive information, were transferred to the Siemens model 7570-C mainframe computer of the Centraal Digitaal Rekencentrum of the Rijksuniversiteit Gent, Belgium,

where they were stored in an adapted version of the Biological DataBase Manager (BIODBM, Pot *et al.*, unpublished). Numerical analysis was performed as described previously (Kersters and De Ley, 1975) on points 7-374 of each interpolated trace. The similarity between all possible pairs of traces was expressed by the Pearson product moment correlation coefficient (r). Strains were clustered by using the unweighted pair group average linkage method (Sokal and Sneath, 1963).

RESULTS

General features of the cell envelope protein profiles

The cell envelope protein profiles consisted of 30-40 discrete protein bands in the molecular weight range of 14.0-100.0 K (Fig. 1). In the 20.0-55.0 K region, a very limited number (2-3) of heavily stained (major) protein bands were present in the patterns of almost all strains investigated (see also Dijkshoorn *et al.*, 1987).Thirteen slab gels, each carrying 16 lanes, including 5 molecular weight reference lanes, were compared in a numerical analysis. Reproducibility of the cell envelope protein patterns of strain number 24, included once in each gel, was $r > 0.96$, i.e. in the range of the reproducibility levels found earlier (Pot *et al.*, 1989; Vandamme *et al.*, 1989). The reproducibility of the 65 low molecular weight marker lanes was found to be $r > 0.93$. This relatively low value was partly due to the use of a linear background subtraction procedure. The Pearson product moment correlation coefficient includes in its calculation the common area of background. In contrast to traces of bacterial protein patterns, no common area of background remained present in the molecular weight marker traces after linear background subtraction. As a consequence, an analysis based on such traces, will show an increased discrimination, resulting in lower similarity values. Also, the presence of only 7 molecular weight protein bands, compared to 30-40 bands present in a bacterial protein pattern of a bacterial extract, will tend to lower the similarity values calculated between traces with a small shift in one or more homologous protein bands.

Numerical analysis

An initial analysis was performed using the pattern between positions 7 and 374 of each trace, corresponding to the protein profile after omission of the stacking gel-running gel interface and of the gel front, carrying the tracking dye. From the resulting dendrogram 17 clusters could be delineated at

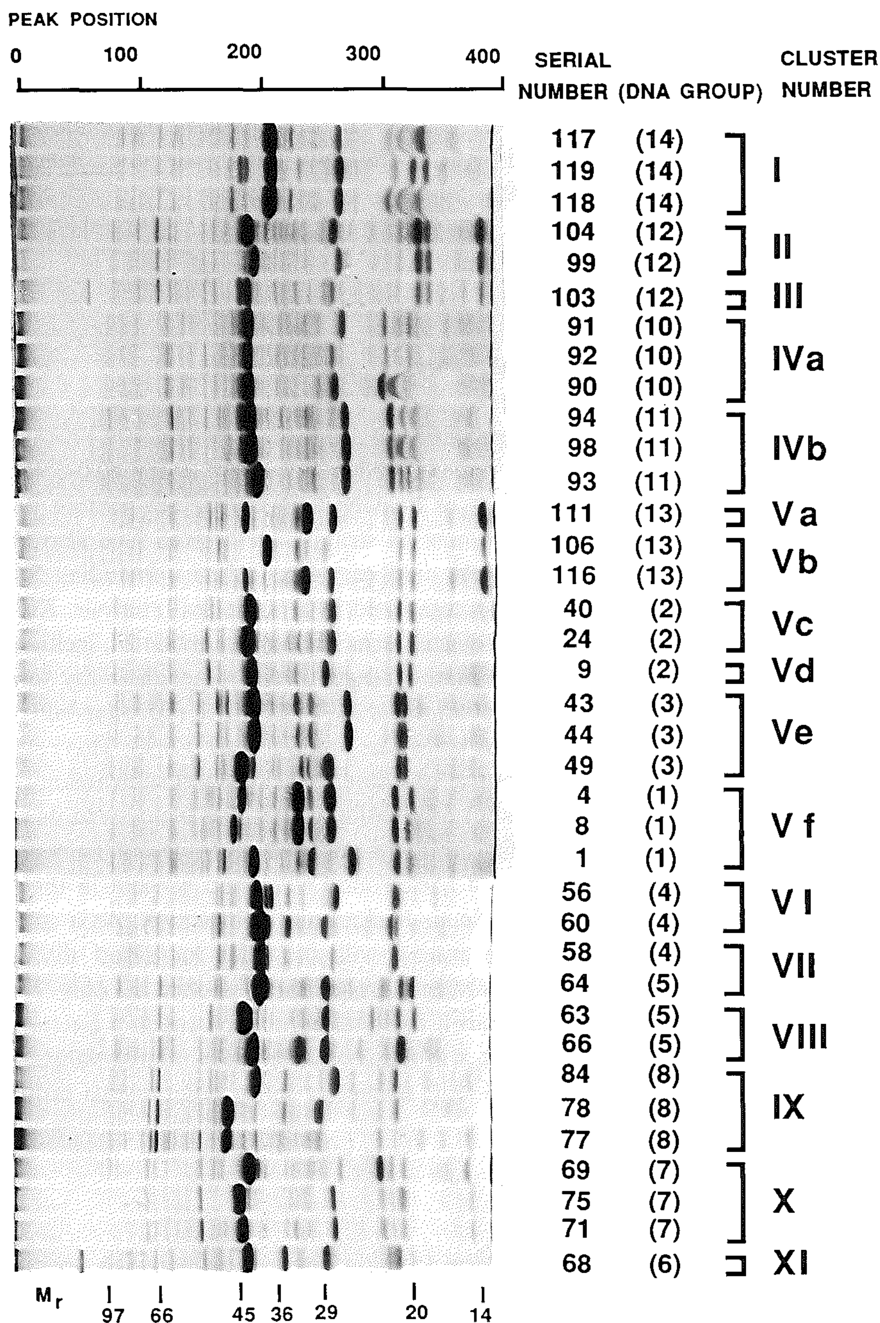

Fig. 1. Cell envelope protein SDS-PAGE profiles of *Acinetobacter* strains of different DNA groups. Strains are designated by serial numbers of Table 1. Scale 0-400, peak positions of densitometric track; M_r, molecular weight markers.

r = 0.62. Only three of these clusters correlated with existing DNA groups, i.e. DNA group 2 (including two strains of DNA group 1) and DNA groups 4 and 14; all the representatives of the other DNA groups were scattered over the electrophoretic clusters (data not shown). Visual inspection and comparative plotting of the cell envelope protein profiles revealed that (i) the electrophoretic clusters were mainly correlated with the matching of major protein bands (data not shown), (ii) within several DNA groups, variation in the position of major protein bands was observed (DNA groups 5, 7 and 8, Fig. 1) and (iii) the banding pattern between position 7 and 150 contained probably the most reliable information with respect to DNA group representation, as this zone was seen to be free of major protein bands for all profiles investigated. Figure 2 shows the resulting dendrogram of the numerical analysis of positions 7-150, containing proteins with molecular weight > 55.0 K.

Relation of the reduced protein profiles to DNA groups

At r = 0.61, eleven clusters were distinguished in the dendrogram shown in Figure 2.

Cluster I. All strains of DNA group 14, linked at r = 0.70. These strains were also linked in the analysis of the complete profiles (points 7-374) at r = 0.66.

Cluster II. This cluster consisted of five clinical isolates from Sweden belonging to DNA group 12 and linked at r = 0.77. Cluster II was linked to cluster I at r = 0.57.

Cluster III. In this cluster the type strain of DNA group 12 (*A radioresistens* IAM 13186) was linked to a culture collection strain of DNA group 12 at r = 0.66. Cluster III was linked to clusters I and II at r = 0.41.

Cluster IV. This cluster consisted of the three strains of DNA group 10 (subcluster IVa) and of the six strains of DNA group 11 (subcluster IVb). The two subclusters were linked at r = 0.70.

Cluster V. This cluster, representing 55% of the strains investigated, consisted of strains belonging to DNA groups 1, 2, 3 and 13. They were linked at r = 0.64. At a cutting level of r = 0.70, six subclusters (Va to Vf) were seen. Subcluster Va contained four DNA group 13 strains and one strain of DNA group 2 (*A. baumannii*). Subcluster Vb contained five strains of DNA group 13, including its reference strain (ATCC 17903), and also one strain of DNA group 2. Subcluster Vf contained 23 strains of DNA group 2 which in a previous epidemiological study were allocated to provisional protein profile groups A and B1-2 (Table 1). Subcluster Vd contained six strains of epidemiologically unrelated strains of DNA group 2, including the type strain of *A. baumannii* and the reference strain for DNA group 2 used in this study.

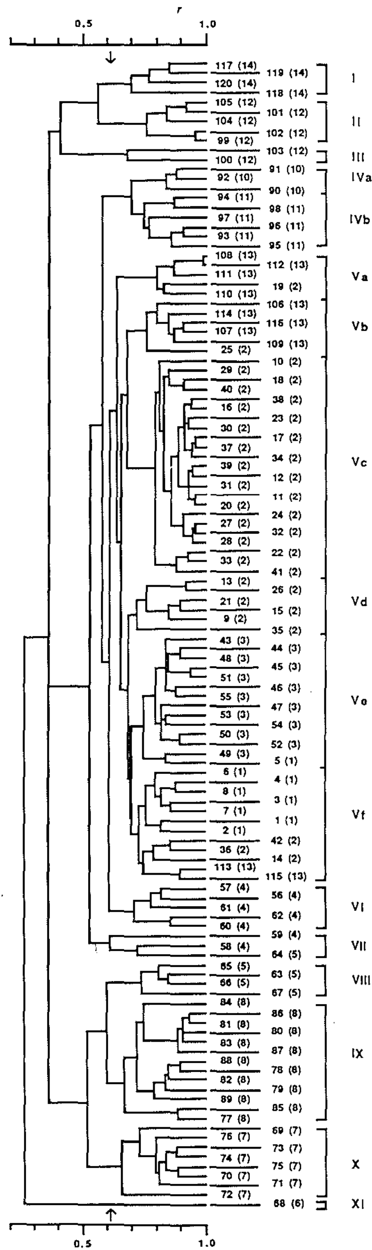

Fig. 2. Dendrogram of numerical analysis of positions 7-150 of cell envelope protein profiles of *Acinetobacter* strains.
Horizontal axis: r = Pearson product moment correlation coefficient.
Vertical axis: strains, designated in alternated position by serial numbers (Table 1) and DNA groups in parentheses. Clusters are coded by Roman numerals. The arrows mark the cutting level (r =0.61).

Subcluster Ve contained 14 strains, all belonging to DNA group 3 and one strain of DNA group 1. Strains allocated earlier to provisional profile group D (Table 1) were all found in this subcluster. Subcluster Vf contained all remaining strains of DNA group 1 (*A. calcoaceticus*), three strains of DNA group 2 and two strains of DNA group 13.
Cluster VI. Five strains of DNA group 4 (*A. haemolyticus*), including the type strain, were linked at r = 0.72 in this cluster.
Cluster VII. This small cluster contained two strains of DNA group 4 and one strain of DNA group 5 linked at r = 0.62. In the analysis of the complete profile, all strains of DNA group 4 were found in one major cluster at the level r = 0.50. Strains of provisional protein profile group E (Table 1) grouped in this major cluster.
Cluster VIII. This cluster contained four strains out of five of DNA group 5 (*A. junii*), linked at r = 0.71.
Cluster IX. All strains of DNA group 8 (*A. lwoffii*) were linked at r = 0.67 in this cluster. Strains allocated to the provisional profile F group (Table 1) grouped in this cluster.
Cluster X. This cluster contained all strains of DNA group 7 (*A. johnsonii*), linking at r = 0.67.
Cluster XI. The single strain of DNA group 6 was the only member of this cluster.
Representative strains of each DNA group, shown in Figure 1, were applied to gels according to the arrangement of clusters of the dendrogram of Figure 2. It can be seen that profiles consisted of several heavily and intermediately stained bands (molecular weight 14.0-55.0 K) and of many minor protein bands. For several DNA groups, the variation of the heavily stained bands was limited (e.g. DNA group 3 in the region 14.0 - 20.0 K, Fig. 1); for other DNA groups the variability was considerable (e.g. DNA groups 5, 8 and 13 in the region 45.0 - 25.0 K, Fig. 1). In contrast, the presence (and variation) of major bands in the upper part of the profile was low. This resulted in more homogeneous banding patterns within each DNA group as well as between some specific DNA groups (e.g. DNA groups 1, 2, 3 and 13, Fig. 1).

DISCUSSION

Theoretical aspects of DNA hybridization versus SDS-PAGE of cell envelope proteins

DNA pairing has been used extensively for classification of bacteria, mainly for two reasons. Firstly, it gives an estimate of the overall similarity of the nucleotide sequences of genomic DNA and, secondly, the DNA relatedness

data show discontinuities which may be used to delineate taxonomic entities. Whole cell protein electrophoretic analysis was used by others in combination with DNA-DNA relatedness studies to elucidate finer inter- and intraspecific relationships (Owen and Jackman, 1982; Pot *et al.*, 1989; Vandamme *et al.*, 1989). SDS-PAGE of cell envelope proteins implies the comparison of a limited number of genes which have been transcribed under different conditions of regulation, as can be deduced from the presence of a number of major protein bands in the electrophoretic profiles of cell envelopes. Therefore, it is not to be expected that the cell envelope protein profile analysis would completely agree with the genomic information determined by DNA-DNA hybridization.

In epidemiological studies, protein profiles are often compared by visual inspection and this approach was followed in several studies on *Acinetobacter* (Dijkshoorn *et al.*, 1987, 1989). In one of these studies (Dijkshoorn *et al.*, 1987), 43 out of 78 strains were thus allocated to provisional groups A-F (Table 1). Most strains of these groups also clustered together in the present numerical analysis, confirming the usefulness of the visual analysis of some groups. As many other strains of the same study could not be classified (i.e. the strains marked as 'un', Table 1), computer-assisted comparisons were required for objective grouping of large numbers of strains.

Numerical analysis of cell envelope protein profiles

It has been observed in other taxonomic studies (Costas *et al.*, 1987; Holmes *et al.*, 1988; Owen *et al.*, 1988a, 1988b, 1989) that strains belonging to different well-established species were not always differentiated into their respective species when their complete protein electrophoretic pattern was included in the numerical analysis. The formation of protein electrophoretic types was overcome by omitting the major protein band region, resulting in clear differentiation of the respective species (Costas *et al.*, 1987; Holmes *et al.*, 1988; Owen *et al.*, 1988a, 1988b, 1989). However, the variability of the major protein band region, appeared useful for grouping and identification at infraspecific level. From our results the same conclusions hold for the analysis of electrophoretic fingerprints of cell envelopes. The use of the nearly complete protein profile (points 7-374 of the densitometric track), revealed groups, which were mainly associated on the basis of matching of heavy bands. When the heavy band region was excluded from the analysis, the correlation with the DNA-groups (Tjernberg and Ursing, 1989) improved considerably.

The relation of protein electrophoretic clusters to DNA groups

Of the twelve DNA groups represented by more than one strain, groups 7, 8 and 14 completely corresponded to electrophoretic clusters X, IX and I, respectively (Fig. 2). Three DNA groups (4, 5 and 12) were each split into two clusters, indicating the greater variability of protein patterns in these groups. Cluster IV consisted of the two DNA groups 10 and 11. Both groups differed in the biotyping scheme of Bouvet and Grimont (1987) only by the outcome of the glucose O/F test. All strains of DNA groups 1, 2, 3 and 13 were found in one electrophoretic cluster V. Only the strains of DNA group 3 grouped together in one of the six subclusters. These findings were in accordance with the observations that DNA groups 1, 2, 3 and 13 are more related to each other than to other DNA groups (Tjernberg and Ursing, 1989), and that groups 1, 2 and 3 show a high degree of phenotypical similarity (Bouvet and Grimont, 1986, 1987).

Practical implications

SDS-PAGE of cell envelope proteins has proven to be successful in epidemiological investigations of *Acinetobacter* strains using the entire protein profile (Dijkshoorn *et al.*, 1987, 1989). The results show that the method can be used in epidemiological investigations without any reference to DNA groups or nomenspecies. Previous studies have shown that relationships between bacteria based on the numerical analysis of whole cell protein electrophoretic patterns agree well with DNA hybridization data (KestersS and De Ley, 1975; Owen and Jackman, 1982) and may be useful to identify well-established species or even strains. This may prove to be rewarding for routine identification as (i) SDS-PAGE is easier accomplished than DNA-DNA hybridization and, (ii) results are more quickly obtained. A biochemical scheme for the identification of *Acinetobacter* strains to the DNA group level (Bouvet and Grimont, 1986, 1987) comprises many tests and is not yet current practice. Therefore, in the present study, correlation of SDS-PAGE cell envelope profiles and DNA-DNA hybridization was investigated for *Acinetobacter*. It appeared that correlation of cell envelope protein profiles and DNA hybridization was not satisfactory when the entire profile was used. The correlation was improved considerably when a restricted part of the profile was used. It is concluded that several DNA groups may be identified by this method, provided that the procedure is standardized, and appropriate reference strains are included.

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CHAPTER 8

GENERAL DISCUSSION

The significance of *Acinetobacter* strains as nosocomial pathogens is well documented. Relatively little is known, however, about the epidemiology of these micro-organisms in the hospital environment. The purpose of this thesis was to develop methods to differentiate *Acinetobacter* strains and, using these methods, to elucidate their niches and mode of transmission. Several studies were performed, which were aimed to answer the following questions:

- Which methods can be used to establish identity or non-identity of strains and how useful are these methods in hospital epidemiology?
- Where do strains reside in patients and in the hospital environment, and how are they transmitted?
- What is the relationship between the cell envelope protein profiles, obtained by SDS-PAGE, and the recently described DNA hybridization groups (genospecies)?
- To which of the DNA hybridization groups do nosocomial strains belong?

In the following paragraphs the results of the studies are discussed.

Typing methods

Two typing methods were developed to differentiate strains: typing on the basis of cell envelope protein SDS-PAGE and typing on the basis of growth on carbon sources. The results of the present thesis rely heavily on SDS-PAGE protein typing. Many studies have shown that this method is useful to study relationships between and within bacterial species [11, 19, 23, 25]. In taxonomical studies, usually whole cell preparations were used to differentiate micro-organisms. In epidemiological studies, outer membrane fractions were used frequently and a few major protein bands in the outer membrane appeared useful to distinguish strains of several species, e.g. *Neisseria meningitidis* and *Haemophilus influenzae* [2, 21]. In our studies, cell envelopes [20] were used and the entire profiles of strains, consisting of 30-40 protein bands

of varying staining intensities were compared visually. Profiles of individual strains were well reproducible. The overall-variation between strains was, generally, considerable. No differences were seen, however, between strains from common epidemiological origin. *Acinetobacter* isolates, obtained during a hospital outbreak, were not only of the same protein profile, but also of the same (unique) antibiotic resistance pattern. In the first two studies of the thesis, strains were allocated to provisional groups on the basis of similarity of protein profiles. Later, however, it appeared that the patterns were generally too heterogeneous to classify these absolutely by visual inspection. The method of cell envelope protein profile typing was, however, useful to type strains relatively during epidemiological studies in specific situations.

The second typing method, based on the growth of strains on different carbon sources, was relatively simple to perform. It was investigated, whether the method was practicable to differentiate strains. When applied to compare strains from three hospitals, the method allowed the discrimination between the three sites. There was, however, a considerable overlap between the strains. One cannot but conclude that the carbon source growth assay in the present form is of limited value in epidemiological studies.

At a later stage of the present investigation, a new taxonomic subdivision of the genus *Acinetobacter* was introduced by Bouvet and Grimont [6]. Twelve groups (genospecies) were established by the use of DNA-DNA hybridization, eleven of which could be identified by biochemical tests. Four new species were described, *A. baumannii* (DNA group 2), *A. haemolyticus* (DNA group 4), *A. junii* (DNA group 5) and *A. johnsonii* (DNA group 7) and the descriptions of *A. calcoaceticus* (DNA group 1) and *A. lwoffii* (DNA group 8) were emended. Moreover, a biotyping scheme was developed for the most prevalent (geno)species, *A. baumannii* [7]. In cooperation with these investigators, a study was performed [9] which is not included in this thesis. The purpose of this study was to compare the methods of phenotypical identification to genospecies level and biotyping [6, 7] with the methods of phage typing [35, 36] and cell envelope protein profile typing (chapter 2). Results of the various methods were consistent for strains from common origin. When strains were identified to genospecies level, biotyping allowed the differentiation of several types within two of the genospecies. Protein profile typing allowed differentiation of strains within other genospecies as well. Protein profile and phage typing were more discriminatory than biotyping and, therefore, seemed more appropriate to establish identity or non-identity of strains. However, as 49 out of 120 strains could not be typed by phages, the value of phage typing was limited.

Epidemiology

Acinetobacters are ubiquitous micro-organisms and are readily cultivated from soil, water and sewage [3, 24, 37]. They have also been found in the hospital environment [28]. The bacteria have frequently been isolated from human sources, including the skin, saliva and the conjuntiva and from all kinds of clinical specimens, e.g. wound material, sputum or cerebrospinalis fluid [5, 14, 16]. Their presence in clinical samples is more often associated with colonization than with infection. However, the bacteria are increasingly involved in serious and fatal opportunistic infections [5]. Hospital outbreaks of acinetobacters are particularly seen in departments with severely ill patients, e.g. a neurosurgical department [13] and intensive care units [29, 30]. Hospital infections have been related to the presence of acinetobacters on the skin of patients and hospital staff [12, 17] and on medical devices, including resuscitation devices and humid parts of the respiratory equipment [10, 15, 30, 34]. As the organisms were frequently isolated from wet sites, the humid environment was presumed to be an important habitat and in a study, directed to moist sites in a burn unit, the bacteria were found in wet mattresses. Allen and Green [1] cultivated the bacteria from air samples and proposed an airborne mode of spread.

In spite of the many data, the reservoirs and route of transmission of acinetobacters in the hospital environment are not well known. This may be explained by the sometimes poor characterization of isolates. For example, in one study, no typing data were presented [29], while strains in other studies were merely differentiated on the basis of the antibiotic resistance pattern [10] and a few additional biochemical tests [17]. In our investigations a typing system was developed to differentiate isolates. The purpose was to contribute to the prevention of spread of the bacteria in the hospital environment. The epidemiological studies are discussed below.

At the beginning of the project, contacts were made to establish the presence of acinetobacters in several hospitals. As a result, many isolates were obtained from different patients and hospital environments. Many of the strains were isolated by an enrichment procedure [33]. This involved inoculation of an acetate-mineral medium with a specimen, followed by vigorous aeration during incubation at 30°. These conditions favoured selective outgrowth of acinetobacters [3]. Acinetobacters involved in nosocomial infection usually produce acid from glucose [5]. Therefore, strains of this phenotype were regarded as the most relevant in our studies.

The bacteria were found in different body sites of the patients. Clinical isolates were obtained from the lower respiratory tract, from urine, wounds, blood and cerebrospinal fluid. In one study (chapter 3) samples were taken

from ten different sites of the skin and mucosal surfaces. Bacteria of one protein profile were found in humid and in more 'dry' sites of the skin, for example in the axilla and on the forehead. They were also cultivated from swabs of the mucosal surfaces, i.e. the nose, the throat or the anus. In some patients, the bacteria were observed in up to eight or nine of the sampled body sites. This shows that large areas of the skin and mucosal surfaces may be colonized. A strain could persist in a patient for many days (6-46 days). Epidemiological samples were usually taken after detection of a strain in clinical specimens of a patient. Therefore, the study did not allow to establish whether colonization of skin and external mucosal surfaces preceded or followed after infection. In some patients (chapter 3), the strains were exclusively found at the site of infection, for example in a wound or in the urinary tract. In another study, bacteria of a common pattern were found in samples from the skin and external mucosal surfaces without further evidence of infection (chapter 5).

The studies showed, that *Acinetobacter* strains may be acquired by patients in the hospital. In one study, strains of a given pattern were found in several patients and cross-infections were substantiated. In another study, performed in a community hospital, a strain of a specific antibiotic resistance and protein pattern was shown to have spread among patients of the intensive care unit. The period between admission of the patients to the ward and their acquisition of this strain varied greatly (1-19 days). In a prospective study in another hospital, two different strains appeared to have spread successively in the intensive care unit. The first strain was predominantly found in clinical specimens. The second strain was only observed in samples from the skin and mucosal surfaces, which were taken for epidemiological reasons. This shows that strains may spread among patients without being noticed by routine bacteriology.
In the period of study it was also investigated, whether there was a relationship between the use of antibiotics and acquisition of acinetobacters. The results did, for the time being, not allow to draw definite conclusions.

In order to elucidate their mode of spread, the presence of acinetobacters in the environment of patients was investigated during two hospital outbreaks. Epidemiological strains, identified by their protein profile, were found in samples from the humid environment, including a slop basin, sinks and a cleaning cloth. It should be mentioned that these strains were also present in 'dry' sites, e.g. on the floor, on cupboards and in dust. In one study, the bacteria were found in an air sample. In the other study, no acinetobacters were grown from the (relatively few) air samples. Samples from skin of the hospital staff were negative.

From their presence in humid and dry sites of the biotic and abiotic environment one must conclude that the bacteria may occupy many different niches. A number of properties of acinetobacters might explain their survival in these environments and their emergence as nosocomial pathogens i.e. their ability to persist in the dry state [1], to resist antibiotics [5] and detergents [12], to use a wide variety of organic nutrients [4] and to adhere to surfaces and epithelial cells [27].

On the basis of the foregoing and data from literature [1, 12, 34] the following epidemiological model is constructed:
Acinetobacters may be introduced into departments of severely ill patients by occasional carriers and may spread from these individuals into the ward. Susceptible patients acquire the bacteria along multiple routes: from the hands of the nursing staff, from contaminated medical devices and from the air or by contact with environmental reservoirs. The bacteria may colonize the skin and mucosal surfaces of patients without being noticed. Ultimately, the increased prevalence of the bacteria confers a high risk of infection in susceptible patients.
To prevent their spread, the presence of glucose-acidifying acinetobacters in clinical samples from the intensive care department should be monitored carefully, even in cases of low quantities or mixed cultures. In case of an increase in the number of isolations, several measures should be taken, including the tracing of carriers, barrier nursing and strict adherence to hand washing and house cleaning procedures. In case of epidemics, more drastic measures have to be considered, e.g. isolation of carriers and closing of the department for a general disinfection.

Classification and identification

Several studies have shown, that the genus *Acinetobacter* is biochemically and genetically heterogeneous [4, 18, 24]. Clear criteria for the differentiation of several species have, for a long time, not been available. Recently, different groups (genospecies, DNA groups) within the genus were established by the use of DNA-DNA hybridization [6, 8, 31]. Identification of strains according to this new taxonomic classification may confer problems in daily routine for several reasons. Firstly, the DNA groups can only be differentiated by DNA-DNA hybridization or by the use of many biochemical tests [6, 7, 8, 31, 32]. These methods are not currently used. Secondly, several of the DNA groups, established in separate studies [8, 31], have been designated by identical numbers and this may be a source of confusion. It has also been shown [31] that the species *A. radioresistens* defined by Nishimura et al. [22] corresponds to DNA group 12 described by Bouvet and Grimont [6].

The present investigation was started before the new classification of DNA groups was known. In the first two studies, strains were allocated to provisional groups on the basis of visual inspection of the cell envelope protein profiles. In a later study, a few of these groups appeared to correspond with specific DNA groups. This showed that strains of several DNA groups, i.e. groups 3 and 4 (*A. haemolyticus*) may be identified by visual analysis of profiles. Generally, however, a considerable heterogeneity of profiles was observed among strains and a satisfactory, absolute classification did not seem possible by visual inspection.

To obtain a more objective classification of profiles, use was made of densitometry and numerical analysis. In a study comprising a collection of 120 strains, it was investigated whether the protein profile groups (clusters) thus obtained, corresponded to groups determined on the basis of DNA hybridization of the strains. When the entire protein profile was used in numerical analysis, clustering was based on the heavily stained protein bands in the profiles. The position of these 'major' bands appeared to vary within strains of a given DNA group and, therefore, clusters did not correspond to DNA groups. The correlation between protein profiles and DNA groups improved when the analysis was based on a restricted part of the profile. This part, which comprised 'minor' protein bands of relatively high molecular weight, could be used for identification of strains of several DNA groups. However, all strains of DNA groups 1 (*A. calcoaceticus*), 2 (*A. baumannii*), 3 and 13 could not be separated on the basis of the restricted profile and formed a single protein profile cluster. These groups share many biochemical characteristics [6] and exhibit a comparatively high degree of DNA relatedness [31]. The relatively high degree of relatedness between these groups at different levels of genetic information, i.e. the genome, the cell envelope proteins and the biochemical behaviour, supports the suggestion of Tjernberg and Ursing [31] to regard DNA groups 1-3 and 13 as one species, *A.calcoaceticus*.

Kersters and De Ley [19] and Owen and Jackman [23] have reported that SDS-PAGE protein profiles agree well with DNA-DNA hybridization data of bacterial strains. In these studies, whole cell preparations were used for electrophoresis. The lack of agreement between the entire protein profiles and DNA-hybridization data of strains, as observed in this thesis, might be due to the use of cell envelope fractions. These fractions comprise relatively many copies of specific protein molecules as can be deduced from the occurrence of a number of major bands in the protein profiles. Numerical analysis showed that these major bands varied between strains of several DNA groups and hampered the identification of strains to these DNA groups. On the other hand, this major band variation seems useful to differentiate strains within DNA groups, which is important in epidemiological typing.

Strains described in chapter 2 were identified at genospecies (DNA group) level by the use of the biochemical system of Bouvet and Grimont [6, 7] and by DNA-DNA hybridization according to the methods of Tjernberg and Ursing [31, 32]. This allowed us to compare the results of the two different identification systems [9; (chapter 7)]. For 54 out of 64 strains, results of the methods were in agreement. Ten strains (no. 31, 34, 36, 59, 62, 63, 64, 65, 67 and 68, chapter 2) by DNA hybridization identified to DNA groups 1, 3 and 13, were not identified to their corresponding group by phenotypical methods. This shows, that phenotypical identification may sometimes fail in the determination of the DNA group of a strain.

Species involved in nosocomial infections

Many studies, including the present investigation, have shown that *Acinetobacter* strains involved in nosocomial infection [14, 26] frequently belong to the glucose-acidifying variety. Glucose non-acidifying strains (*A. lwoffii sensu lato*) have been associated with incidental infections but seem to be less often involved in epidemical outbreaks. Several of the recently described genospecies (DNA groups) comprise glucose-acidifying strains and it may be asked, to which DNA groups nosocomial strains belong. In several studies [7, 38], strains associated with nosocomial infections and hospital outbreaks were identified as *A. baumannii* (DNA group 2). In our studies, many clinical isolates, including strains involved in epidemical outbreaks also belonged to this (geno)species [9; (chapter 7)]. It is concluded that *A. baumannii* is a species of clinical-epidemiological significance.

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SUMMARY

In recent years, acinetobacters have manifested as bacteria which can spread epidemically in hospitals and may cause severe infections. Treatment of the infections may confer problems, because strains are often resistant to antibiotics. The investigation of the present thesis was aimed to elucidate the sources and the mode of transmission of the bacteria in the hospital environment.

As acinetobacters are widespread in nature, typing methods are needed to differentiate strains in epidemiological studies. Two typing methods were developed for the present investigation. One method was based on the visual analysis of cell envelope protein profiles of strains obtained by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE); the other method was based on the comparison of growth of strains on several carbon sources. The cell envelope protein typing method appeared useful to relatively type strains from a restricted geographical area, e.g. a hospital, and to trace identical strains. In comparison with the methods of biotyping and phage typing, which had been developed elsewhere, the cell envelope protein typing appeared highly discriminatory. Moreover, the method allowed profile determination of all strains, while the other methods did not. By the second typing method, the carbon source growth assay, it was possible to distinguish between groups of strains from common epidemiological origin, but not between individual strains. Therefore, this method seemed to be of limited value in epidemiological studies.

Several studies were performed to investigate the epidemiology of *Acinetobacter* in the hospital. The bacteria were found in clinical specimens from various sites of the body of patients. After their primary isolation in clinical specimens, bacteria of the same protein profile were also cultivated from samples from the skin and mucous membranes of these patients. In some patients, large areas of the skin and mucosal surfaces seemed to be colonized. A strain could persist in a patient for several weeks.
On the basis of the analysis of protein profiles and epidemiological data it was established that patients could acquire *Acinetobacter* strains in the hospital. In one study, several cross-infections were substantiated. In another study it was found that a strain had spread epidemically among patients of an intensive

care unit of a community hospital. The period between admission of the patients to the ward and acquisition of the strain varied from 1-19 days. In another hospital two different strains had spread successively among patients. One strain was only observed in epidemiological samples from the skin and mucosal surfaces and not in clinical specimens.
In order to elucidate their mode of spread, the environmental presence of the bacteria was investigated during two hospital outbreaks of *Acinetobacter*. Remarkably, the epidemical strains were not only found in moist sites, but also in an air sample and in dry sites, for example on the floor and in dust. The strains were not found on the skin of hospital staff.
These studies show that the bacteria may reside in ecologically different niches, both in patients and in the inanimate environment. It is therefore presumed that the patients acquire the bacteria from several sources and along different routes. A strict adherence to hygienic rules as well as the tracing and isolation of carriers are advocated for the purpose of prevention of nosocomial spread.

In the course of the investigation a new taxonomic classification of *Acineto-bacter* strains was introduced. The groups of this classification were established on the basis of DNA-DNA hybridization data of strains. In a final study of the present thesis, we investigated whether cell envelope protein profiles could be used to identify strains according to this classification of DNA groups. The profiles of 120 strains were determined densitometrically and subjected to numerical analysis. When a restricted part of the protein profile was used in the numerical analysis, a number of protein profile clusters corresponded to specific DNA groups. Thus, this part of the profile, comprising minor bands of relatively high molecular weight, appeared useful for identification of strains to the level of these DNA groups. The remaining part of the protein profile, which comprised major protein bands, seemed to be more useful to distinguish strains of the same DNA group. The latter is important in epidemiological studies.

The studies allowed to deduce, that strains of DNA group 2 (*A. baumannii*) are highly prevalent in infections and nosocomial outbreaks. It is concluded that this group is of particular clinical-epidemiological significance.

SAMENVATTING

Acinetobacters hebben zich de laatste jaren gemanifesteerd als bacteriën die zich epidemisch kunnen verspreiden binnen ziekenhuizen en daarbij ernstige infecties kunnen veroorzaken. Behandeling van de infecties kan problemen geven omdat de stammen dikwijls resistent zijn voor antibiotica. Het in dit proefschrift beschreven onderzoek had tot doel inzicht te verkrijgen in de bronnen en verspreidingswegen van de bacteriën in het ziekenhuis.

Aangezien acinetobacters wijdverspreid in de natuur voorkomen, is er behoefte aan typeringsmethoden, waarmee stammen bij epidemiologisch onderzoek kunnen worden onderscheiden. Voor dit doel werden twee typeringsmethoden ontwikkeld. De ene methode was gebaseerd op de visuele vergelijking van celenvelop-eiwitprofielen van stammen verkregen door natriumdodecylsulfaat-polyacrylamide gel electroforese (SDS-PAGE); de andere methode was gebaseerd op vergelijking van de groei van stammen op een aantal koolstofbronnen. De celenvelop-eiwittypering bleek bruikbaar om stammen uit een beperkt geografisch areaal, bijvoorbeeld een ziekenhuis, relatief te typeren en om identieke stammen op te sporen. Bij vergelijking met de elders ontwikkelde methoden van biotypering en faagtypering bleek de celenvelop-eiwittypering een relatief hoog onderscheidend vermogen te hebben. Bovendien kon van alle stammen het eiwitprofiel worden bepaald, terwijl bepaling van biotype en faagtype van alle stammen niet mogelijk bleek. De tweede typeringsmethode, de koolstof-assimilatie methode, was wel bruikbaar voor onderscheid van groepen van stammen met een gemeenschappelijke herkomst, maar niet voor onderscheid van individuele stammen. Het nut van deze methode voor epidemiologisch onderzoek leek dan ook beperkt.

Er werden verschillende studies uitgevoerd om inzicht te krijgen in de epidemiologie van *Acinetobacter* in het ziekenhuis. De bacteriën werden bij patiënten gevonden in klinische materialen afkomstig van verscheidene delen van het lichaam. Na primaire isolatie van de bacteriën in klinische monsters werden bacteriën van hetzelfde eiwitprofiel bovendien gevonden in monsters van huid en slijmvliezen van deze patiënten. Bij sommige patiënten schenen grote delen van het huid-en slijmvliesoppervlak te zijn gekoloniseerd. De stammen persisteerden in sommige patiënten gedurende tientallen dagen.

Door analyse van eiwitprofielen en epidemiologische gegevens kon worden vastgesteld, dat patiënten met *Acinetobacter* stammen kunnen worden besmet in het ziekenhuis. In een studie werden diverse kruisbesmettingen tussen patiënten aannemelijk gemaakt. Uit een andere studie bleek dat een stam zich epidemisch had verspreid onder patiënten van een intensive care unit van een streekziekenhuis. De tijd tussen opname van de patiënten op de afdeling en besmetting met de stam varieerde van 1-19 dagen. In een ander ziekenhuis bleken zich op de intensive care unit achtereenvolgens twee verschillende stammen onder patiënten te hebben verspreid. Een hiervan werd slechts op huid en slijmvliezen en niet in klinische materialen opgemerkt.
Om de wijze van verspreiding op te helderen werd tijdens twee verheffingen van acinetobacters onderzoek gedaan naar de aanwezigheid van de bacteriën in de omgeving van patiënten. Opmerkelijk was, dat de epidemische stammen niet alleen in vochtige plaatsen, maar ook in een luchtmonster en op droge plaatsen, bijvoorbeeld op de vloer en in stof, werden aangetroffen. Huidmonsters van het ziekenhuispersoneel waren negatief.
Uit deze studies blijkt, dat de bacteriën in ecologisch verschillende plaatsen aanwezig kunnen zijn, zowel binnen een patiënt als in de niet-levende omgeving. Het is derhalve waarschijnlijk, dat patiënten de bacteriën uit verscheidene bronnen en langs verschillende wegen kunnen verwerven. Om verspreiding te voorkomen wordt een strikte naleving van hygienische maatregelen, alsmede het opsporen en isoleren van dragers bepleit.

In de loop van het onderzoek werd een nieuwe taxonomische indeling voor acinetobacters geintroduceerd, waarbij groepen werden onderscheiden op grond van DNA-DNA hybridizatie gegevens van stammen. Onderzocht werd, of de celenvelop SDS-PAGE eiwitprofielen kunnen worden gebruikt om stammen te identificeren volgens deze indeling van DNA groepen. Voor dit doel werden de profielen van 120 stammen door densitometrie bepaald en aan numerieke analyse onderworpen. Als een beperkt deel van het eiwitprofiel voor numerieke analyse werd gebruikt, correspondeerden een aantal clusters met bepaalde DNA groepen. Dit deel van het profiel, dat bestond uit relatief dunne bandjes van hoog molecuulgewicht, bleek dus bruikbaar voor identificatie van stammen tot het niveau van deze DNA groepen. Het overige deel van het eiwitprofiel, dat meer dikke eiwitbanden omvatte, scheen meer bruikbaar voor onderscheid van stammen binnen een DNA groep. Dit laatste is van belang voor epidemiologisch onderzoek.

Uit de studies kon worden afgeleid, dat stammen van DNA groep 2 (*A. baumannii*) het meest frequent betrokken waren bij infecties of epidemieën. Geconcludeerd wordt, dat vooral deze groep van klinisch-epidemiologische betekenis is.

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CURRICULUM VITAE

De schrijfster van dit proefschrift is op 8 april 1944 in Zoetermeer geboren. In 1959 behaalde zij haar diploma MULO-B, in 1965 verkreeg zij het diploma Algemeen Klinisch Analist (SAL) en werkte daarna als analist in de Academische Ziekenhuizen van Leiden en Utrecht. Sinds 1967 werkt zij als docent in het Hoger Laboratorium Onderwijs. In de periode van 1975-1981 combineerde zij deze funktie met een studie biologie aan de Rijksuniversiteit in Utrecht, welke cum laude werd afgerond. Met steun van het Praeventiefonds bewerkte zij binnen het Academisch Ziekenhuis Rotterdam Dijkzigt van 1984 tot heden in een deeltijdfunktie het onderzoek van dit proefschrift.

CIP-GEGEVENS KONINKLIJKE BIBLIOTHEEK, DEN HAAG

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Dit proefschrift kwam tot stand op de Afdeling Bacteriologie van het Academisch Ziekenhuis Rotterdam Dijkzigt. Van 1 september 1984 tot 1 september 1989 werd het onderzoek gesubsidieerd door het Praeventiefonds.

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