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Presence of the *ica* operon in clinical isolates of *Staphylococcus epidermidis* and its role in biofilm production

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ABSTRACT

Staphylococcus epidermidis is an important cause of catheter-associated infections, which are attributed to its ability to form a multilayered biofilm on polymeric surfaces. This ability depends, in part, on the activity of the *icaADBC* locus and the *icaR* gene, which are involved in the production of the polysaccharide intercellular adhesin (PIA) that is functionally necessary for cell-to-cell adhesion and biofilm accumulation. The present study determined: (1) the prevalence of the *icaADBC* operon in *S. epidermidis* isolates from catheter-related and other nosocomial infections; (2) the correlation between the presence of this operon, biofilm production and resistance to antibiotics; (3) the expression of *ica* genes and biofilm production; and (4) the genetic relatedness of the isolates. The results showed that *icaRADBC* was present in 45% of the isolates included in the study, and that such isolates were significantly more resistant to the main antibiotics tested than were *ica*-negative isolates. The presence of the entire cluster did not always correlate with biofilm production, determined under different culture conditions, but there was evidence to suggest a correlation when at least two genes (*icaAD*) were co-transcribed. Eight of 18 *ica*-positive isolates had the entire operon in the same restriction fragment after pulsed-field gel electrophoresis, but the isolates were not clonal. Estimation of genetic relatedness indicated that *ica*-positive *S. epidermidis* isolates belonged to different lineages, distributed in only one of two major clusters, with a genetic distance of *c*. 0.12.

Keywords Biofilm, catheter-associated infections, coagulase-negative staphylococci, *ica* operon, *Staphylococcus epidermidis*

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INTRODUCTION

Coagulase-negative staphylococci, particularly *Staphylococcus epidermidis*, are the most frequent causes of implanted medical device- or catheter-related infections, as well as nosocomial bacteraemia [1]. Despite the generally low virulence of *S. epidermidis*, it is now recognised as a potential pathogen because of its ability to form biofilms following primary bacterial attachment to specific polymeric surfaces, with subsequent multilayered cluster accumulation or intercellular adhesion. This multi-step mechanism is considered to be one of the major factors involved in the pathogenicity of catheter-related infections caused by *S. epidermidis* [2]. Investigation of the second stage of biofilm formation has demonstrated that cell aggregation and biofilm accumulation are mediated by the products of the chromosomal *ica* gene locus, which comprises four intercellular adhesion genes (*icaA*, *icaB*, *icaC* and *icaD*) and one regulator gene (*icaR*), which seems to function as a repressor [3–6]. These genes influence the production of polysaccharide intercellular adhesin (PIA), which is a partially *N*-acetylated linear β-1–6-linked glucosaminoglycan. PIA is involved in haemagglutination [4,7,8] and also acts as an intercellular adhesin on glass and (probably)

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other hydrophilic surfaces [9]. The role of PIA in resistance to phagocytosis and other host defence mechanisms is currently being investigated.

The amount of biofilm production, analysed phenotypically, is also influenced by changing environmental conditions, in that stimuli such as sub-inhibitory concentrations of tetracycline and quinopristin–dalfopristin, as well as high temperature, anaerobiosis, ethanol stress and osmolarity, are known to enhance *ica* transcription and biofilm formation. Complex systems, such as an alternative sigma factor σ^B, *icaR*, an ethanol-mediating activator of *ica* operon expression, or insertion and excision of the insertion sequence IS*256* at specific hot spots of the *icaA* and *icaC* genes, contribute to this complex regulation system [5,10–12].

The present study analysed 40 epidemiologically unrelated *S. epidermidis* isolates, collected over a 6-month period, in order to determine: (1) the presence of the *icaADBC* operon in *S. epidermidis* isolates from catheter-related and other nosocomial infections; (2) the correlation between the presence of this operon, biofilm production and resistance to antibiotics; (3) the expression of *ica* genes and biofilm production; and (4) the localisation of the *ica* operon on the chromosome and the genetic relatedness of the isolates.

MATERIALS AND METHODS

Bacterial isolates

The 40 *S. epidermidis* isolates included in the study were from catheter-related and other nosocomial infections. Only isolates for which ≥15 CFU were obtained by semiquantitative culture [13,14] were included in the study. Colonisation was defined as isolation of the same organism from at least two segments of the catheter or from the tip, with the latter applying only for patients with a previous history of sepsis or with a positive blood culture. The isolates were collected from three Italian laboratories in a 6-month period during 2000, and were single isolates from different patients (Table 1). *S. epidermidis* ATCC 35984 (RP62A) was used as a positive control for biofilm production, as an *ica* operon positive control, and for the amplification probes for the *ica* operon.

All isolates were identified with the API-Staph System (bioMérieux, Marcy l'Etoile, France). MICs of oxacillin, ampicillin, gentamicin, vancomycin, teicoplanin, ciprofloxacin, levofloxacin, co-trimoxazole, erythromycin, tetracycline and rifampicin were determined according to NCCLS guidelines [15]. The methicillin-susceptible *S. aureus* strain ATCC 29213 was used as a control for antimicrobial susceptibility tests.

Quantitative and qualitative determination of biofilm production

Two different methods were used to assay biofilm production: a spectrophotometric assay (quantitative method), and observation of slime production (qualitative method). For the quantitative measurement, each isolate was grown in brain heart infusion broth and tryptone soya broth (Oxoid, Basingstoke, UK), and in a chemically defined medium (CDM), prepared as described previously [16], except that $MgSO_4$ was added before the dissolution of the remaining components, and L-cystine was dissolved in 2–3 drops of 5 M NaOH before addition to the medium. These assays were performed in microtitre plates as described previously [6]. Each reported value was the average of 12 measurements at 490 nm. Qualitative determination was performed with a Congo Red agar assay [17]. Agar plates were incubated at 37°C for 24 h, and then for an additional 24 h at room temperature. Black colonies were considered to be positive variants, while red colonies were considered to be negative.

PCR

Genomic DNA was isolated from each strain as described previously [18]. Amplification of the five genes of the *ica* operon was by PCR with specific primers (Table 2) designed from the GenBank published sequence (accession number U43366). Amplification mixes contained 10 mM Tris-HCl pH 8.8, 1.5 mM $MgCl_2$, 150 mM KCl, Triton X-100 0.1% v/v, 1 U of *Taq* polymerase (DyNAzyme II DNA polymerase; Finnzymes, Oy, Espoo, Finland), 200 μM each dNTP and 1 μL of genomic DNA. PCR conditions comprised an initial 5-min denaturation step at 94°C, followed by 30 cycles of 1 min at 93°C, 30 s at 53°C (58°C for *icaC*) and 2 min at 68°C, with a final annealing step of 1 min at 53°C (or 58°C) and extension for 10 min at 68°C.

For amplification of the entire *ica* operon, a long PCR was performed with primers *icaR* up and *icaC* down (Table 2). Amplification was carried out with Takara La *Taq* polymerase (BioInc. OTSU, Shiga, Japan), used according to the manufacturer's instructions, with an initial 5-min denaturation step at 94°C, followed by 30 cycles of 1 min at 93°C, 30 s at 50°C and 15 min at 68°C, with a final annealing step of 1 min at 50°C and elongation for 10 min at 68°C. The predicted amplicon size was 4013 bp.

DNA sequence analysis

The nucleotide sequences of PCR products were obtained by the method of Sanger *et al.* [19]. Sequencing was performed on a Li-Cor automated sequencing system (Li-Cor Biotechnology, Lincoln, NE, USA) with infrared dye-labelled sequencing primers. The sequencing reactions were performed with a ThermoSequenase fluorescent-labelled primer cycle sequencing kit (Amersham Bioscience, Chalfont St Giles, UK), used according to the manufacturer's instructions.

Study of expression

RNA extraction

Cultures were grown in brain heart infusion broth to mid-log phase, as determined by spectrophotometric growth rate analysis at 600 nm. Cells were centrifuged at 4000 *g* for 30 min and

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Table 1. *Staphylococcus epidermidis* isolates (n = 40) included in the study

Laboratory number	Isolate number	Species	Hospital	Italian city	Sources
1	1CT	*S. epidermidis*	University Polyclinic	Catania	Catheter tip
2	2CT	*S. epidermidis*	University Polyclinic	Catania	Catheter tip
3	3CT	*S. epidermidis*	Vittorio Emanuele	Catania	Catheter tip
4	4CT	*S. epidermidis*	Cannizzaro	Catania	Catheter tip
5	5CT	*S. epidermidis*	University Polyclinic	Catania	Catheter tip
6	29 CAN	*S. epidermidis*	Cannizzaro	Catania	Catheter tip
7	63 CAN	*S. epidermidis*	Cannizzaro	Catania	Catheter tip
8	82CCH	*S. epidermidis*	Ferrarotto	Catania	Catheter tip
9	86CCH	*S. epidermidis*	Ferrarotto	Catania	Catheter tip
10	1ACLB	*S. epidermidis*	Vittorio Emanuele	Catania	Catheter tip
11	11CLB	*S. epidermidis*	Vittorio Emanuele	Catania	Catheter tip
12	24CLB	*S. epidermidis*	Vittorio Emanuele	Catania	Catheter tip
13	AN3	*S. epidermidis*	Umberto I	Ancona	Catheter tip
14	AN6	*S. epidermidis*	Umberto I	Ancona	Catheter tip
15	AN8	*S. epidermidis*	Umberto I	Ancona	Catheter tip
16	AN10	*S. epidermidis*	Umberto I	Ancona	Catheter tip
17	RO1	*S. epidermidis*	Gemelli Polyclinic	Rome	Catheter tip
18	RO3	*S. epidermidis*	Gemelli Polyclinic	Rome	Catheter tip
19	RO4	*S. epidermidis*	Gemelli Polyclinic	Rome	Catheter tip
20	RO5	*S. epidermidis*	Gemelli Polyclinic	Rome	Catheter tip
21	71CCH	*S. epidermidis*	Ferrarotto	Catania	Wound swab
22	75CCH	*S. epidermidis*	Ferrarotto	Catania	Pleural drain
23	80CCH	*S. epidermidis*	Ferrarotto	Catania	Blood
24	6CT	*S. epidermidis*	University Polyclinic	Catania	Bronchial aspirate
25	150D	*S. epidermidis*	University Polyclinic	Catania	Blood
26	RO15	*S. epidermidis*	Gemelli Polyclinic	Rome	Blood
27	RO16	*S. epidermidis*	Gemelli Polyclinic	Rome	Blood
28	RO17	*S. epidermidis*	Gemelli Polyclinic	Rome	Blood
29	3CLB	*S. epidermidis*	Vittorio Emanuele	Catania	Catheter tip
30	$4CLB_2$	*S. epidermidis*	Vittorio Emanuele	Catania	Catheter tip
31	RO6	*S. epidermidis*	Gemelli Polyclinic	Rome	Catheter tip
32	RO9	*S. epidermidis*	Gemelli Polyclinic	Rome	Catheter tip
33	RO10	*S. epidermidis*	Gemelli Polyclinic	Rome	Catheter tip
34	AN1	*S. epidermidis*	Umberto I	Ancona	Catheter tip
35	42CAN	*S. epidermidis*	Cannizzaro	Catania	Catheter tip
36	RO19	*S. epidermidis*	Gemelli Polyclinic	Rome	Blood
37	RO20	*S. epidermidis*	Gemelli Polyclinic	Rome	Blood
38	163D	*S. epidermidis*	University Polyclinic	Catania	Bronchial aspirate
39	161POL	*S. epidermidis*	University Polyclinic	Catania	Bronchial aspirate
40	19CLB	*S. epidermidis*	Vittorio Emanuele	Catania	Blood

Table 2. Primers and their sequences used in the study

Primer	Sequence	Fragment size (bp)	Reference
icaR forward[a]	5′-TACTGTCCTCAATAATCCCGAA	453	This study
icaR reverse[a]	5′-GGTACGATGGTACTACACTTGATG		
icaA forward[a]	5′-TCTCCCCCTTATTCAATTTTCT	1466	This study
icaA reverse[a]	5′-CGATACAATACATCCAAAATACTC		
icaD forward[a]	5′-CAGACAGAGGCAATATCCAAC	225	This study
icaD reverse[a]	5′-ACAAACAAACTCATCCATCCG		
icaB forward	5′-ATGGCTTAAAGCACACGACGC	526	[6]
icaB reverse	5′-TATCGGCATCTGGTGTGACAG		
icaC forward[a]	5′-ATCATCGTGACACACTTACTAACG	934	This study
icaC reverse[a]	5′-CTCTCTTAACATCATTCCGACGCC		
mecA forward	5′-GGTCCCATTAACTCTGAAG	1040	[21]
mecA reverse	5′-AGTTCTGCAGTACCGGATTTTGC		

[a]Primer designed from published GenBank sequence (accession number U43366).

then resuspended in 200 μL of diethylpyrocarbonate (DEPC)-treated H_2O containing 30 μL of DPEC 0.5% v/v (diluted in H_2O), 100 μL of lysostaphin (50 μg/mL) and 100 μL of lysozyme (100 μg/mL). After incubation at 37°C for 30 min, 1 mL of Trizol reagent (Gibco BRL, Paisley, UK) was added and incubation was continued for a further 5 min. Following incubation, 200 μL of DEPC-treated chloroform was added, and mixed by agitation; the mixture was incubated for 5 min, and then centrifuged at 10 000 *g* for 15 min. Following centrifugation, 1 mL of ice-cold isopropanol was added to the supernatant and the mixture was incubated at room temperature for 30 min. After centrifugation at 12 000 *g* for 15 min, the pellet was resuspended in 50 μL of DEPC-treated H_2O and stored at −20°C. Genomic DNA was removed by treatment with RNase-free DNase I (Ambion, Austin, TX, USA), and the residual RNA concentration was determined spectrophotometrically. Each extracted RNA sample was also used as a template in a PCR assay to confirm the absence of DNA contamination.

RT-PCR

RT-PCR was performed with a two-step procedure in which *c*. 200 ng of RNA was added to a mix comprising 50 mM Tris-HCl, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol pH 8.3, 200 μM each dNTP, 0.2 μM primers (as described for PCR), and 200 U of M-Mulv reverse transcriptase (BioLabs, Beverly, MA, USA). cDNA synthesis was performed for 10 min at 58°C for the *icaABC* genes, and at 54°C for the *icaRD* and *pta* genes, followed by incubation at 37°C for 20 min. Each RT-PCR assay amplified the *pta* gene (a house-keeping gene) as an internal positive control, with the use of primers described previously [20].

Pulsed-field gel electrophoresis

Preparation of chromosomal DNA for pulsed-field gel electrophoresis (PFGE) was as described previously [21,22]. DNA was digested with 20 U of *Sma*I (New England Biolabs, Beverly, MA, USA) and analysed on a Gene-Navigator

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apparatus (Pharmacia, Uppsala, Sweden) with the following PFGE parameters: 20 h at 8°C in 0.5 × TBE buffer at 6 V/cm, with a forward pulse time of 5 s for 5 h, 10 s for 5 h, 22 s for 5 h, and 35 s for 5 h. A λ-ladder PFGE marker was included in each run as a molecular size standard. Genetic relatedness, based on the *Sma*I PFGE profiles, was calculated by unweighted pair-group method analysis (UPGMA), with arithmetic averages based on the Dice coefficient [23–25].

Hybridisation

PFGE fragments were transferred to Hybond N^+ nylon membranes (Amersham Bioscience) with a vacuum blotting apparatus (Pharmacia), used according to the manufacturer's instructions, and were hybridised with three different probes (the *icaA* gene, the entire *ica* locus amplicon, and the *mecA* gene) labelled with the non-radioactive ECL system (Amersham Bioscience). The *mecA* probe was obtained by PCR with the *mecA* forward and *mecA* reverse primers (Table 2) [21]. All probes were purified with a PCR-product purification kit (Qiagen, Valencia, CA, USA).

RESULTS

Antibiotic susceptibility

The isolates included in the study were subdivided by PCR into *ica*-positive and *ica*-negative groups. Isolates possessing the *ica* operon were more resistant to all classes of antibiotic tested than were *ica*-negative isolates. In particular, there were significant differences in resistance to oxacillin, gentamicin, ciprofloxacin, levofloxacin, co-trimoxazole and erythromycin (Table 3). All isolates showed a very high percentage of resistance to ampicillin and were uniformly susceptible to vancomycin and teicoplanin.

Table 3. Antibiotic susceptibilities of the *Staphylococcus epidermidis* isolates, subdivided with respect to the presence or absence of the *ica* operon

Antibiotic	*ica*-positive strains 18/40 (45%)	*ica*-negative strains 22/40 (55%)	Significance
Oxacillin			
Range	1–256	≤ 0.25–128	
MIC_{50}	32	4	
MIC_{90}	128	64	
%R	88	45.4	$p < 0.05$
Gentamicin			
Range	≤ 0.25–256	≤ 0.25–128	
MIC_{50}	64	0.25	
MIC_{90}	128	64	
%R	88	31.8	$p < 0.001$
Ampicillin			
Range	256 to > 1024	4 to > 1024	
MIC_{50}	1024	1024	
MIC_{90}	> 1024	> 1024	
%R	100	95.4	NS
Vancomycin			
Range	2–8	2–8	
MIC_{50}	4	4	
MIC_{90}	8	8	
%R	0	0	
Teicoplanin			
Range	1–32	1–32	
MIC_{50}	8	8	
MIC_{90}	32	16	
%R	0	0	
Ciprofloxacin			
Range	0.12 to > 256	0.12 to > 256	
MIC_{50}	8	0.25	
MIC_{90}	> 256	16	
%R	66.6	18.2	$p < 0.01$
Levofloxacin			
Range	0.12–32	0.12 to > 256	
MIC_{50}	2	0.25	
MIC_{90}	16	4	
%R	38.8	4.5	$p < 0.05$
Co-trimoxazole			
Range	≤ 0.25–32	≤ 0.25–16	
MIC_{50}	8	≤ 0.25	
MIC_{90}	16	0.5	
%R	61.1	4.5	$p < 0.001$
Erythromycin			
Range	≤ 0.12–1024	0.12–1024	
MIC_{50}	1024	0.5	
MIC_{90}	1024	1024	
%R	72.2	27.3	$p < 0.05$
Tetracycline			
Range	≤ 0.5–128	0.5–64	
MIC_{50}	2	0.5	
MIC_{90}	4	32	
%R	11.1	13.6	
Rifampicin			
Range	≤ 0.25 to > 256	≤ 0.25–1	
MIC_{50}	≤ 0.25	≤ 0.25	
MIC_{90}	> 256	≤ 0.25	
%R	33.3	0	

NS, not significant; R, resistance.

ica operon organisation; quantitative and qualitative determination of biofilm production

The presence of the *icaADBC* genes, including the regulatory gene *icaR*, was demonstrated by amplification of the corresponding fragments in 18 (45%) of 40 isolates. All amplicons were of the expected sizes, and IS*256* insertions were not detected (Table 4). The *ica*-positive isolates were distributed among both catheter-related isolates ($n = 11$ isolates) and those from other nosocomial infections ($n = 7$). Among the 18 *ica*-positive isolates, 15 produced a biofilm, as measured by both methods used in this study, while three isolates possessing the entire operon were negative for biofilm production. Qualitative (Congo Red agar assay) and quantitative determinations of biofilm production performed in triplicate showed a good correlation; all strains positive by one test were also positive by the other (Table 4).

Quantitative spectrophotometric determination demonstrated that cultural conditions influenced the production of biofilm (Table 5), and it was possible to subdivide the isolates growing in CDM into strong or weak producers, according to their ability to adhere to the base of the plastic wells, by measuring the absorbance at 490 nm [26]. Only the positive control (strain RP62A) had

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Table 4. Correlation between the presence of the *ica* operon (PCR amplicons; bp) and biofilm production in brain heart infusion (BHI) broth, as measured with a spectrophotometric assay (positive cut-off ≥0.12 at 490 nm) and slime production on Congo Red agar (CRA)

		PCR fragments (bp)					Biofilm production	
Isolate	Source	*icaR*	*icaA*	*icaB*	*icaC*	*icaD*	CRA	BHI broth
RP62A		453	1466	526	934	225	+	1.29
1	Catheter	453	1466	526	934	225	+	0.28
2	Catheter	None	None	None	None	None	No	NP
3	Catheter	453	1466	526	934	225	No	NP*
4	Catheter	453	1466	526	934	225	+	0.12
5	Catheter	453	1466	526	934	225	No	NP*
6	Catheter	None	None	None	None	None	No	NP
7	Catheter	None	None	None	None	None	No	NP
8	Catheter	453	1466	526	934	225	No	NP*
9	Catheter	453	1466	526	934	225	+	0.12
10	Catheter	453	1466	526	934	225	+	0.4
11	Catheter	None	None	None	None	None	No	NP
12	Catheter	None	None	None	None	None	No	NP
13	Catheter	453	1466	526	934	225	+	0.14
14	Catheter	None	None	None	None	None	No	NP
15	Catheter	None	None	None	None	None	No	NP
16	Catheter	None	None	None	None	None	No	NP
17	Catheter	None	None	None	None	None	No	NP
18	Catheter	None	None	None	None	None	No	NP
19	Catheter	453	1466	526	934	225	+	0.16
20	Catheter	None	None	None	None	None	No	NP
21	Other	453	1466	526	934	225	+	0.13
22	Other	453	1466	526	934	225	+	0.13
23	Other	453	1466	526	934	225	+	0.21
24	Other	453	1466	526	934	225	+	0.14
25	Other	453	1466	526	934	225	+	1.12
26	Other	None	None	None	None	None	No	NP
27	Other	453	1466	526	934	225	+	0.12
28	Other	453	1466	526	934	225	+	0.20
29	Catheter	453	1466	526	934	225	+	0.16
30	Catheter	None	None	None	None	None	No	NP
31	Catheter	None	None	None	None	None	No	NP
32	Catheter	None	None	None	None	None	No	NP
33	Catheter	None	None	None	None	None	No	NP
34	Catheter	None	None	None	None	None	No	NP
35	Catheter	453	1466	526	934	225	+	0.6
36	Other	None	None	None	None	None	No	NP
37	Other	None	None	None	None	None	No	NP
38	Other	None	None	None	None	None	No	NP
39	Other	None	None	None	None	None	No	NP
40	Other	None	None	None	None	None	No	NP

NP*, *ica*-positive biofilm non-producer; NP, biofilm non-producer.

the same absorbance value for all three media; for all other isolates, biofilm production was enhanced in the CDM. Based on the above criteria, there were 13 strong producers, four weak producers (with two at the lower limit), and one negative isolate.

Hybridisation with *ica* operon and *mecA* probes

Southern blotting and hybridisation with a probe for the entire *ica* operon demonstrated that the *ica* operon was localised in the *Sma*I PFGE fragment XI in eight (44%) isolates (Fig. 1). The corresponding PFGE profiles showed that the isolates did not belong to a single clone (data not shown). The remaining ten isolates carried the

Table 5. Quantitative determination of biofilm production under different culture conditions (brain heart infusion (BHI) broth, tryptone soya broth (TSB) and chemically defined medium (CDM))

Isolate	Biofilm production in BHI	Biofilm production in TSB	Biofilm production in CDM
RP62A	1.29	1.225	1.78
1	0.28	0.08	1.46
3	0.06	0.10	0.19
4	0.12	0.36	0.67
5	0.07	0.07	0.05
8	0.07	0.10	0.12
9	0.12	0.23	0.36
10	0.4	0.09	0.175
13	0.14	0.08	0.43
19	0.16	0.07	0.25
21	0.13	0.10	0.26
22	0.13	0.10	0.27
23	0.21	0.13	0.65
24	0.14	0.12	0.44
25	1.12	0.22	0.12
27	0.12	0.15	0.59
28	0.20	0.06	0.56
29	0.16	0.07	0.34
35	0.6	0.98	1.34

Isolates were defined as strong biofilm producers (> 0.24), weak biofilm producers (0.12–0.24) or non-biofilm producers (< 0.12).

operon on four different fragments, namely fragments V (n = 2), X (n = 4), XII (n = 2) and IX (n = 2). Hybridisation with the *mecA* probe showed that 16 of 18 *ica*-positive isolates carried the *mecA* gene, but that this gene was not carried on the same fragment as the *ica* operon (data not shown).

ica operon expression

Expression of *icaADBC* was studied in ten epidemiologically unrelated isolates that were selected for their different ability to form biofilm (including the three negative isolates). Only four isolates showed expression of the *icaADBC* genes (Table 6). All biofilm producers expressed *icaD*; the *icaABC* genes were not expressed by the non-producers (isolates 3, 5 and 8), even though they were *ica*-positive by PCR. Co-expression of *icaAD* seemed to be necessary for a biofilm-positive phenotype. The entire operon was sequenced in these isolates, and no alterations or point mutations were detected.

Genetic relatedness

The 40 isolates clustered into 30 different PFGE profiles, none of which predominated in the sample. The results demonstrated the existence of related PFGE patterns among oxacillin-resistant and *ica*-positive isolates of *S. epidermidis*

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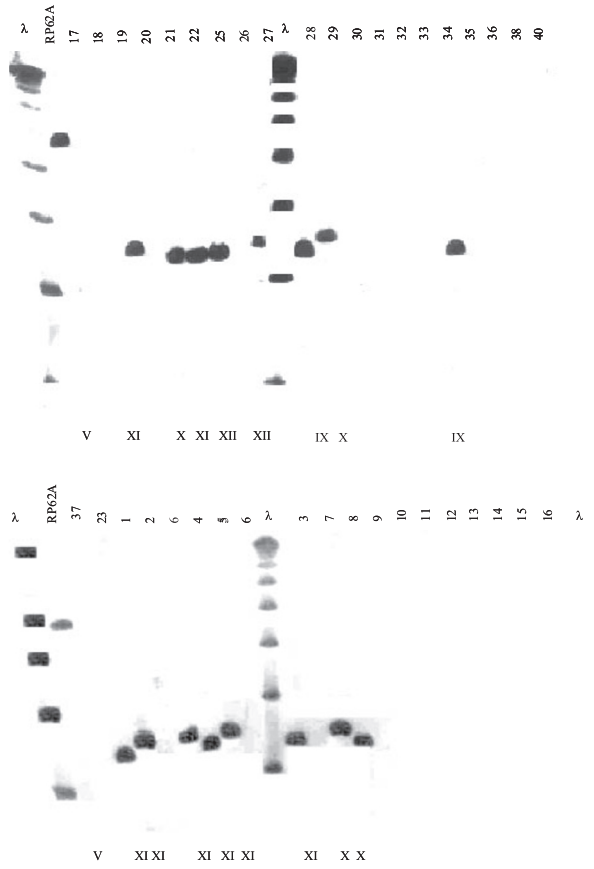

Fig. 1. Southern blot of *Sma*I PFGE fragments from different *Staphylococcus epidermidis* isolates following hybridisation with a probe for the *ica* operon.

Table 6. RT-PCR amplicons (bp) of the *icaABCD* genes produced from a representative sample of ten *Staphylococcus epidermidis* isolates

	Amplicons (bp)			
Isolate	***icaA***	***icaB***	***icaC***	***icaD***
1	1466	526	None	225
3	None	None	None	225
4	1466	526	934	225
5	None	None	None	225
8	None	None	None	225
10	1466	526	934	225
13	1466	526	934	225
21	1466	526	934	225
24	1466	None	None	225
25	1466	526	934	225

from different sources. The isolates could be subdivided into two major clusters with a genetic distance of *c*. 0.12 (clusters A and B), with the *ica*-positive isolates, regardless of whether they were oxacillin-susceptible or oxacillin-resistant, and with the sole exception of isolate 29, grouped in cluster A. In contrast, *ica*-negative isolates were distributed in the two major clusters according to whether they were resistant (cluster A) or susceptible (cluster B) to oxacillin (data not shown).

DISCUSSION

S. epidermidis is a successful coloniser of human skin and mucous membranes, but neither the reasons for this success nor the factors responsible for its change from an innocuous saprophyte to a pathogen are understood precisely. It is currently the most frequent cause of nosocomial sepsis and infections of implanted medical devices, and its pronounced antibiotic resistance presents a major problem, with a high percentage of isolates being multiply resistant [27]. Biofilm formation is considered to be one of the main virulence factors [28], as the most important infections caused by this microorganism are infections involving foreign bodies. Several studies have shown that the *ica* gene locus is always present in sepsis-causing isolates, but is not detectable in saprophytic isolates [28–30]. Biofilm formation in staphylococci is multifactorial, and the ability to form biofilm enhances the ability of strains to survive in normally hostile environments.

In the present study, 45% of *S. epidermidis* isolates from catheter-related and other nosocomial infections were *ica*-positive. The *ica*-positive isolates were also more resistant to antibiotics, thus confirming previous observations [30]. There was a strong association between the presence of the *ica* operon and the presence of the *mecA* gene (16 of 18 isolates), and consequently, with a multiply resistant profile. These results support the idea that the *ica* and *mecA* genes can be considered as important virulence markers for clinically significant staphylococcal isolates; the expression of both gene clusters confers an enhanced survival ability and favours the onset of chronic infections, such as those associated with implanted medical devices. All of the isolates included in the study originated from single or mixed infections, and are probably able to produce adhesins for the host matrix proteins that are adsorbed *in vivo* on to the surface of biomaterials [31–34].

The presence of the entire *icaADBC* gene cluster, including the regulatory gene *icaR*, was

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confirmed by PCR and Southern blot analysis in 18 isolates, but only 15 of these were considered to produce biofilm. In agreement with the results of other studies, biofilm production was highly influenced by cultural conditions, with the presence of glucose 1% w/v in a CDM enhancing the production of biofilm in almost all isolates. All *ica*-negative isolates failed to produce biofilm under the conditions tested in this study, but two of the three *ica*-positive non-biofilm producers became producers in CDM. Southern blot analysis demonstrated that the *ica* operon was generally present on the same PFGE fragment (unlinked to the *mecA* gene) in different isolates, but PFGE analysis showed that most of the isolates belonged to different strains.

Expression of the *icaADBC* operon is essential for the synthesis of polysaccharide intercellular adhesin, which mediates cell-to-cell adhesion and promotes a biofilm-positive phenotype for *S. epidermidis*. The present study analysed the transcriptional activity of *ica* genes in a sample of biofilm-positive and -negative isolates, and demonstrated that *icaD* was always expressed, but that biofilm production occurred only when *icaA* was also expressed. The reasons for the absence of biofilm production in some isolates, despite the presence of the entire *ica* operon, and the lack of transcription of the *icaC* gene in some isolates, considered necessary for PIA elongation, remain unclear. Only one promoter, located upstream of the *icaA* gene, has been mapped in the entire operon [6], and the possibility of sequence alterations and point mutations, perhaps causing loss of function of downstream cistrons, was excluded in these isolates.

In view of these findings, two different hypotheses can be proposed: (1) the overlap of both *icaA/D* and *icaB/C*, together with the observation that the corresponding proteins are encoded by different frames of the same nucleotide sequence, could support the idea of a regulatory mechanism that controls the simultaneous transcription of mRNA from the different *ica* genes; or (2) the *icaR* gene product, together with changes in environmental conditions, could influence transcription of the *ica* operon. The present study demonstrated that the presence of the entire cluster did not always correlate with biofilm production, but there was an indication that such a correlation may occur when at least two genes (*icaAD*) were co-transcribed.

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