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A study of phenotypic variation of *Staphylococcus epidermidis* using Congo red agar

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SUMMARY

This study examines a series of phenotypic variants of *Staphylococcus epidermidis* that were generated from a pair of parent variants, isolated from valvular tissue of a patient with prosthetic valve endocarditis. The variants were initially classified by examining their colonial morphology on Congo red agar. In addition to differences in Congo red binding and colonial morphology, they differed in the expression of several surface components and enzymes. Despite these phenotypic differences, all variants had the same restriction endonuclease profile of plasmid DNA. Examination of a collection of clinical isolates demonstrated that phenotypic variation is a common property of *S. epidermidis*. The ability to express different combinations of surface components and enzymes could contribute to the virulence of *S. epidermidis* strains by enabling these organisms to colonize a range of diverse environments.

INTRODUCTION

In a recent study [1], coagulase-negative staphylococci (CNS) were demonstrated to have the greatest propensity for colonial variation compared with all other species of bacteria isolated from blood cultures. Phenotypic variation in colonial morphology among strains of CNS isolated from humans and experimental animals has been the subject of several other reports [1–10] and has been suggested as a virulence mechanism of these organisms [4, 5, 8]. In this report, we describe the derivation and characterization of a series of phenotypic variants generated from two colonial morphotypes of *Staphylococcus epidermidis* isolated from a patient with native valve endocarditis. The new generation of variants differed from the parents in the range of potential virulence factors expressed.

MATERIALS AND METHODS

Staphylococcal strains

The parent variants (*S. epidermidis*, A-204 and A-205) were isolated from valvular tissue of a patient with native valve endocarditis and have been described previously [11]. Briefly, they differ in haemolysis, adherence, extracellular polysaccharide production, proteolytic and lipolytic activity and pigmentation on Congo red agar, but have the same antibiotic sensitivity profile and both have a

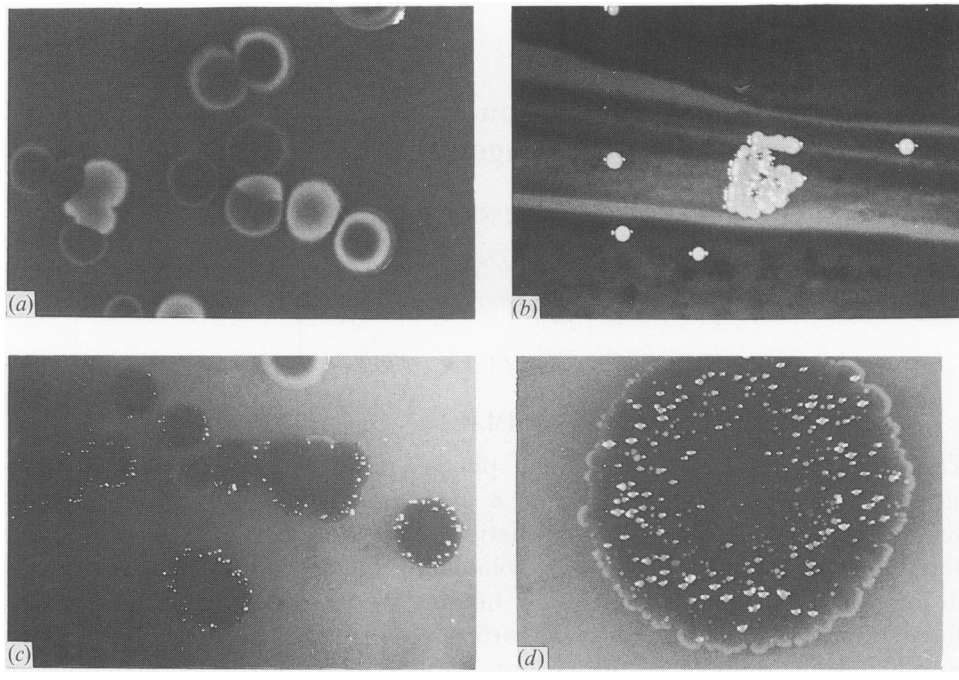

Fig. 1. Four different types of colonial variation. (*a*) Sectored colony in a culture displaying significant variation. (*b*) Crop of pale pink microcolonies superimposed on primary inoculum. (*c*) Highly variable culture showing segmented colonies, 'wart' formation and variant colonial forms. (*d*) Single pale pink colonies superimposed on parent colony.

hydrophobic cell surface. Cellular DNA profiles generated by pulsed field electrophoresis and restriction profiles of plasmid DNA are identical. Both parents have the capacity for phenotypic variation, although one (A-204) gives rise to variants at a greater rate than the other (A-205).

S. epidermidis, strain RP62A (ATCC 35982), a gift from G. Christensen, was used as a control in these experiments. This strain is a strong slime producer [5].

Microbiological media

While investigating the association between Congo red binding and slime production by CNS, we observed that some strains showed marked variation in colonial morphology on Congo red agar (CRA). This medium consisted of 0·003% Congo red in Oxoid blood Agar Base No. 2. Cultures were examined as previously described [11] after incubation for 2 days at 35 °C followed by a further 2 days at room temperature.

Streptococcal salt tolerance broth [12] was prepared without glucose, using Oxoid ingredients.

Selection of sub-populations from parent variants

Variant colonial forms were defined as colonies on CRA that differed morphologically from the parent from which they were originally derived. This definition included segmented colonies (Fig. 1a and 1c). Variants were defined as stable if single colonies retained their morphology after one subculture. A series of

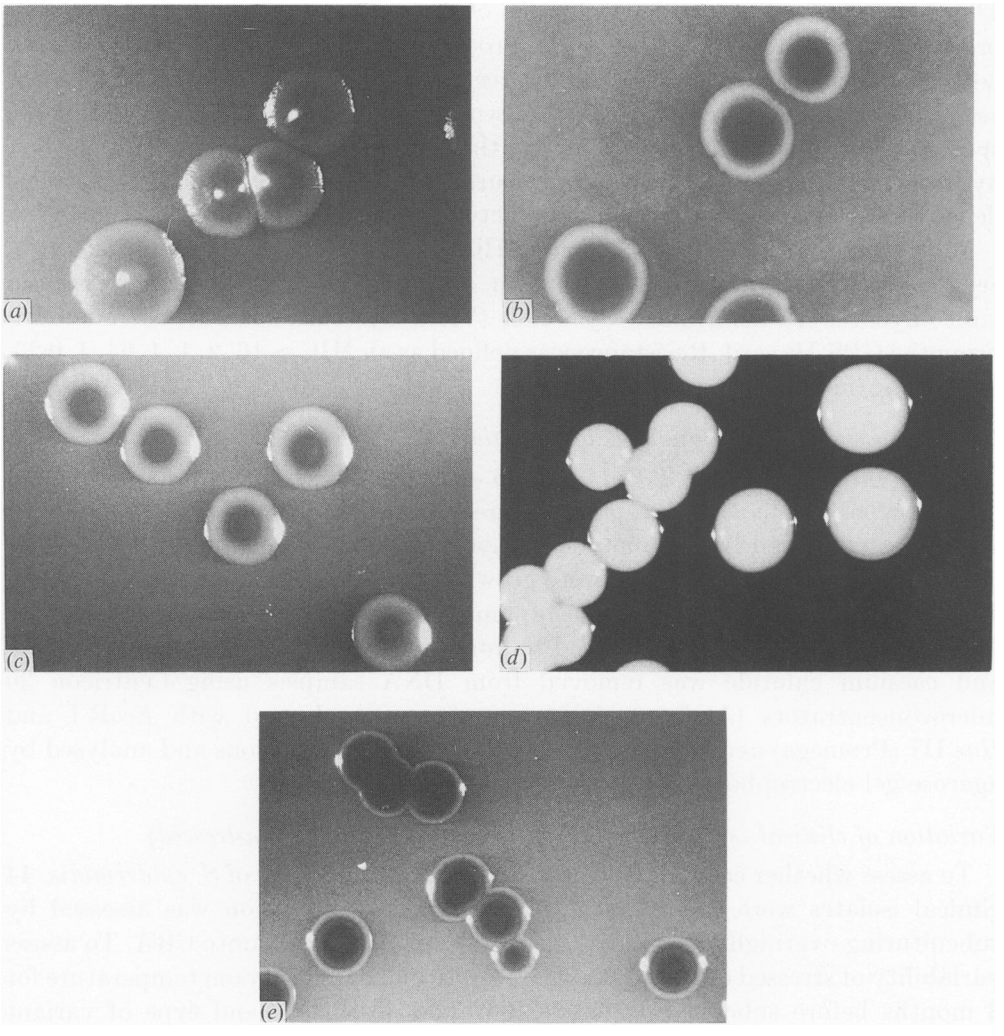

Fig. 2. Five major colonial phenotypes generated from the parent variants, A-204 and A-205; on Congo red agar incubated for 2 days at 35 °C followed by 2 days at room temperature. (*a*) Pink colonies with a central nipple (Type A). (*b*) Dark colonies with a narrow peripheral area which does not take up Congo red (B1). (*c*) Pale colonies with a central dark red region (B2). (*d*) Pale pink amorphous colonies (Type C1). (*e*) Dark red transparent colonies, containing concentric rings of variable colour intensity (Type E).

27 stable variants representing a range of colonial morphotypes was generated from A-204 and A-205, by selecting variant colonies on CRA plates, subculturing segmented colonies – which often yielded up to four colonial morphotypes, enriching for slime-positive or slime-negative variants [5] or subculturing broth cultures that had undergone prolonged incubation. Variants A-364, A-365, A-374 and A-375 were generated by Stephen Pearson by the same procedure.

Characterization of daughter variants

The variants were speciated by both conventional [13, 14] and rapid methods (API Staph and ID 32 Staph; API Systems SA, La Balme Les Grottes, France).

They were also tested for the production of a range of enzymes and extracellular products, including lipase, lecithinase, proteinase and haemolysins as previously described for the parents [11]. Adherence was assessed by the tube adherence assay described by Christensen and co-workers [15], and a modification of the spectroscopic technique described by the same group [16, 17]. Cell surface hydrophobicity was determined by measuring the percentage reduction in optical density after allowing cells to partition between xylene and water phases [11, 18].

Minimal inhibitory concentrations (MICs) of chloramphenicol, ciprofloxacin, erythromycin, gentamicin, nitrofurantoin, oxacillin, penicillin G, tetracycline and vancomycin were determined by Vitek (Vitek Systems, Hazelwood, Missouri) using the GPS-IB card. Resistance was defined as an MIC > 16, 2, 1, 4, 64, 4, 0·25, 8 and 8 μg/ml respectively.

Restriction endonuclease analysis of plasmid DNA

Seven variants (Nos. 1, 2, 8, 11, 13, 25 and 26) representing all major colonial morphotypes and biochemical profiles were selected for plasmid analysis. Plasmid DNA was extracted by the method of Lyon and colleagues [19], except for the following modifications: strains were grown in Circleprep medium (Bio 101 Inc., La Jolla, California), the final lysostaphin concentration was 20 units/ml, 10% Triton X-100 (Selby anax) replaced sodium dodecyl sulphate in the lysis buffer and caesium chloride was removed from DNA samples using Centricon 30 microconcentrators (Amicon). DNA samples were cleaved with *Eco*R I and *Hae* III (Promega) according to the manufacturer's instructions and analysed by agarose gel electrophoresis [20].

Variation of clinical isolates of S. epidermidis *(stressed and unstressed)*

To assess whether colonial variation is a common property of *S. epidermidis*, 44 clinical isolates were examined. The initial rate of variation was assessed by subculturing overnight TSB or salt tolerance broth cultures onto CRA. To assess variability of stressed cells, broth cultures were incubated at room temperature for 3 months before subculturing onto CRA. The proportion and type of variant colonial forms was estimated by counting an appropriate number of well-isolated colonies. In these studies, significant variation was defined as the presence of any sectored colonies or the presence of at least 10% variant colonial forms on CRA plates.

RESULTS

Derivation of daughter variants

Under the conditions used to generate variants, A-204 showed a greater capacity for phenotypic variation than A-205. Thus, of the collection of 27 stable variants, 18 were derived from A-204 and 9 from A-205.

Morphological classification of colonial variants

By examination of colonial morphology on CRA with the aid of a plate microscope, it was possible to define six major colonial morphotypes of stable variants (Fig. 2). Four morphotypes (A, B2, C1 and C2) were pale pink and opaque in overall appearance. Those with a rough texture and a central nipple were classified as Type A. Pale pink, smooth, homogeneous colonies were classified as

C1 (dome-shaped) or C2 (flat and resembling A-205) . Colonies that consisted of a dark red central area surrounded by a wide annular ring of pale pink growth (radius > 20% of the colony diameter) were classified as B2.

The other two morphotypes (B1 and E) were red in general appearance. Colonies resembling A-204 i.e. those with a dark red central area surrounded by a pale narrow peripheral zone (radius < 20% of the diameter of the colony) were defined as B1. Type E colonies were defined as transparent forms with complex morphology, consisting of a series of annular rings differing in pigmentation. The major colonial type of the control strain RP62A showed type E morphology. Although generally stable, the red phenotypes, particularly type E variants, had a greater tendency to undergo colonial variation than the paler morphotypes. The variant collection included 5, 3, 7, 4, 5, and 3 strains of types A, B1, B2, C1, C2 and E respectively.

Although it was possible to classify most variants as one of the major morphotypes, there were some intermediate forms which were difficult to categorize. Moreover, dryness of the agar or heavy inoculation of plates sometimes resulted in altered morphology.

Other types of colonial variation

Another type of colonial variation was sometimes observed when CRA cultures of strain A-205 remained at room temperature for several days beyond the standard 4 day incubation period. Variants appeared as crops of tiny pink colonies superimposed on the primary inoculum (Fig. 1b). Subculture of these forms yielded normal sized colonies belonging to the C1 morphotype.

Cultures of A-204, incubated under the same conditions, sometimes generated numerous tiny colonies superimposed on a red parent colony (Fig. 1c). This phenomenon was also common among other variants and clinical isolates, but was confined to those expressing one of the dark phenotypes. Subculture of these forms yielded normal-sized pale pink dome shaped colonies (type C1).

It should be noted that Congo red agar was used in this study to facilitate the detection of variant forms and sectored colonies. Congo red was not responsible for promoting variation, since variants could also be detected, to some extent, on nutrient agar and other standard laboratory media.

Characterization of variant collection: adherence capacity, haemolysis, biochemical characteristics, extracellular products, relative cell surface hydrophobicity

All variants with the exception of 13 and 14 were identified as *S. epidermidis* by conventional biochemical methods, but they showed a wide range of enzyme activities and adherence capabilities (Table 1). Type A variants characteristically grew in TSB as a heavy deposit beneath a clear supernatant (autoagglutination). All type B1 variants were derived from A-205 and were morphologically and biochemically identical to the other parent, A-204. Most type B2 variants resembled A-204 biochemically, but differed in adherence capacity in addition to colonial morphology. Type E variants were more strongly adherent than all other variants, including the parents.

Type C variants were more diverse than the other types in their enzyme activities, biochemical capabilities and API profiles. Variants 13 and 14 (type C1)

Table 1. *Colonial variation, haemolysis, API profiles, selected enzyme activities, adherence and autoagglutionability of variant collection*

Variant number	Colonial phenotype	Parent	β haemolysis	Lipase and lecithinase	Proteinase	Nitrate reductase	API Staph	Tube adherence	Microtitre adherence	Auto-agglutination
A-204	B1	Parent	−	−	−	+	6 716 131	−	0·29 (−)	−
A-205	C2	Parent	+	+	+	−	6 716 133	+	0·40 (+)	−
1	A	A-204	−	−	−	+	6 716 131	−	0·06 (−)	+
4	A	A-204	−	−	−	+	6 716 131	−	0·04 (−)	+
7	A	A-204	−	−	−	+	6 716 131	−	0·08 (−)	+
10	A	A-204	−	−	−	+	6 716 131	−	0·03 (−)	+
24	A	A-204	−	−	−	+	6 716 131	+	0·18 (−)	−
26	B1	A-205	−	−	−	+	6 716 131	−	0·10 (−)	−
27	B1	A-205	−	−	−	+	6 716 131	−	0·12 (−)	−
A-365	B1	A-205	−	−	−	+	6 716 131	−	0·32 (+)	−
2	B2	A-204	−	−	−	+	6 716 131	+	0·12 (−)	−
6	B2	A-204	−	−	−	+	6 716 131	+	0·13 (−)	−
9	B2	A-204	−	−	−	+	6 716 131	+	0·08 (−)	−
15	B2	A-204	−	−	−	+	6 716 131	+	0·29 (−)	−
21	B2	A-204	−	−	−	+	6 716 131	+	0·13 (−)	−
A-375	B2	A-204	−	−	−	+	6 716 131	+	0·20 (−)	−
25	B2	A-205	−	+	+	−	6 714 173	−	0·09 (−)	−
11	C1	A-205	+	+ + +	+ + +	−	6 714 171	−	0·16 (−)	−
12	C1	A-205	+	+	+	−	6 714 031	−	0·15 (−)	−
13	C1	A-205	−	−	−	+ + +	6 216 000	−	0·03 (−)	−
14	C1	A-205	−	−	−	+ + +	6 006 000	−	0·07 (−)	−
A-364	C2	A-205	+	+ + +	+ + +	−	6 714 173	−	0·22 (−)	−
16	C2	A-204	−	+ +	−	+	6 716 131	−	0·36 (+)	−
17	C2	A-204	−	−	−	+ + +	6 206 112	−	0·02 (−)	−
18	C2	A-204	−	−	−	+ + +	6 206 112	−	0·02 (−)	−
19	C2	A-204	−	−	−	+ + +	6 206 112	−	0·02 (−)	−
3	E	A-204	−	−	−	+	6 716 133	+	1·34 (+ + +)	−
8	E	A-204	−	−	−	+	6 716 131	+	0·76 (+ +)	−
22	E	A-204	−	−	−	+	6 716 131	+	1·21 (+ + +)	−

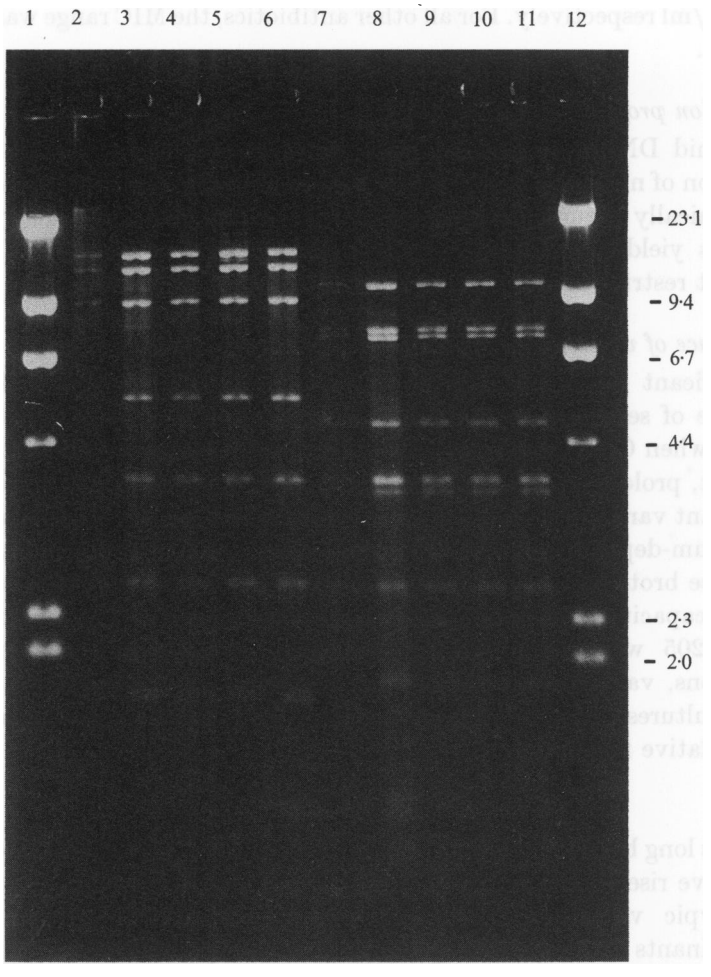

Fig. 3. Restriction endonuclease digestion of plasmid DNA isolated from five variants representing the major colonial morphotypes. Lanes 1 and 12 are molecular weight markers in kilobases (*Hin*dIII digest of λ DNA). Lanes 2 to 6 and lanes 7 to 11 contain digests of variants 8 (type E), 11 (type C1), 25 (type B2), 26 (type B1) and 1 (type A) respectively, cleaved with *Eco*R 1 (lanes 2 to 6) and *Hae* III (lanes 7 to 11).

expressed some enzymes at higher levels than the parents but were biochemically inert with respect to other activities (Table 1).

Contrary to the findings of Freeman and co-workers [21] we found no association between adherence capacity and Congo red binding, under the conditions used in this study. The red phenotypes were not consistently adherent nor were the pink phenotypes always non-adherent. Congo red binding was not associated with a hydrophobic cell surface. With the exception of type A, which became hydrophilic in the late logarithmic phase of growth, all variants expressed a strongly hydrophobic surface throughout the growth cycle.

Minimal inhibitory concentrations (MICs)

Although all variants and parents were categorized as sensitive to nine antibiotics, MICs of vancomycin and chloramphenicol ranged from 0·5–4 μg/ml and

2–16 μg/ml respectively. For all other antibiotics, the MIC range was one doubling dilution.

Restriction profiles of plasmid DNA

Plasmid DNA was isolated from all variants that were examined with the exception of number 13. It is interesting to note that variant 13 was also inactive biochemically despite having large mucoid colonial morphology. The other variants yielded identical profiles after digestion of plasmid DNA with two different restriction endonucleases (*Eco*R I and *Hae* III) (Fig. 3.)

Prevalence of variation among clinical isolates of S. epidermidis

Significant variation in colonial morphology ($\geqslant 10\%$ colonial variation or presence of segmented colonies) was observed in 6 of 44 (14%) *S. epidermidis* strains when CRA subcultures were prepared from overnight broth cultures. In contrast, prolonged incubation of broth cultures of the same strains resulted in significant variation in 68% of subcultures. The type of variation appeared to be a medium-dependent, with TSB favouring the paler morphotypes and salt tolerance broth favouring the expression of darker colonial morphotypes with a greater capacity to absorb Congo red. Similar observations were made when A-204 and A-205 were subjected to prolonged incubation. Under these stressed conditions, variants were apparently produced at random since quadruplicate broth cultures often differed markedly in the type of variants generated and in their relative frequency.

DISCUSSION

It has long been known that pure cultures of CNS on standard laboratory media often give rise to a range of variant colonial forms. Few early studies examined phenotypic variation of these organisms because they were regarded as contaminants of no clinical significance. Recently, CNS have emerged as major nosocomial pathogens and there has been renewed interest in the possible association between their capacity for phenotypic variation and their virulence [4, 7, 9].

From two colonial morphotypes of *S. epidermidis*, isolated from valvular tissue of a patient with endocarditis, we generated a family of variants, whose members differed in the expression of proposed virulence markers such as a high relative cell surface hydrophobicity [22], exopolysaccharide production [10], Congo red binding [21] and various tissue-damaging enzymes and extracellular products [23] as well as MICs of chloramphenicol and vancomycin. All variants from which plasmids could be isolated had identical restriction endonuclease profiles.

Variation in colonial morphology was not confined to the endocarditis variants but was found to be a common property of clinical isolates of *S. epidermidis*, particularly when stressed.

These observations are similar to those of Christensen and colleagues who used a high salt, low glucose medium to detect colonial variants [24]. These authors generated a series of colonial variants differing in slime production and virulence in the rat endocarditis model and also demonstrated a high rate of phenotypic variation among other CNS strains. Variants differing in the degree of resistance

to antibiotics have been detected previously, both *in vitro* [7, 24] and *in vivo* [1, 2, 6].

The genetic mechanisms responsible for the diversity of our variants are unknown, but the observed changes cannot be explained by plasmid loss or gain, since restriction profiles were identical (with the exception of variant 13) from which plasmids could not be isolated. Other possible mechanisms include the movement of transposons or the insertion or deletion of phage DNA [25, 26]. One recent study has demonstrated that a rearrangement of chromosomal DNA, probably via an inversion or insertion, is responsible for alterations in the phenotypic expression of exopolysaccharide and other virulence factors by *Pseudomonas aeruginosa* [27].

It is unknown whether *in vivo* populations of our endocarditis strains are equally capable of generating a highly heterogeneous population of cells with respect to potential virulence determinants and resistance to antibiotics. However, natural populations of pathogenic bacteria rarely contain a homogeneous population of cells, but consist of several distinct clonal lineages, not all of which are equally capable of causing disease [25]. The mechanisms by which this diversity is created are complicated and poorly understood, but there is increasing evidence that bacteria adapt to life *in vivo* by altering the expression of virulence genes [25, 28].

The nature of the small colonies that were observed overgrowing mature colonies after prolonged incubation, is unknown. They appear to be similar to the description of papillae by Hall [29]. Papillae appeared on normal colonies only after nutrient depletion and were the result of an advantageous mutation which enabled the cells to utilize a specific nutrient [29]. Our small colony mutants also appeared only under stress and they reverted to normal-sized colonies when subcultured onto fresh media. The high frequency of variation that occurred in broth cultures after prolonged incubation could be due to a similar mechanism.

In summary, we have shown that *S. epidermidis* strains have a remarkable capacity for phenotypic variation. The relevance of the various phenotypes *in vivo*, and the genetic mechanisms responsible for this variation are being investigated.

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REFERENCES

1. Travis LB, MacLowry JD. Clinically significant differences in antibiograms of morphological variants of blood culture isolates. Diagn Microbiol Infect Dis 1989; **12**: 177–9.
2. Baddour LM, Phillips TN, Bisno AL. Coagulase-negative staphylococcal endocarditis. Arch Intern Med 1986; **146**: 119–21.
3. Franson TR, Sheth NK, Menon L, Sohnle PG. Persistent *in vitro* survival of coagulase-negative staphylococci adherent to intravascular catheters in the absence of conventional nutrients. J Clin Microbiol 1986; **24**: 559–61.
4. Baddour LM, Christensen GD. Prosthetic valve endocarditis due to small colony staphylococcal variants. Rev Infect Dis 1987; **9**: 1168–73.
5. Christensen GD, Baddour LM, Simpson WA. Phenotypic variation of *Staphylococcus epidermidis* slime production *in vitro* and *in vivo*. Infect Immun 1987; **55**: 2870–7.

6. Schwable RS, Stapleton JT, Gilligan PH. Emergence of vancomycin-resistance in coagulase-negative staphylococci. N Engl J Med 1987; **316**: 927–31.
7. Baddour LM, Simpson WA, Weems JJ, Hill MM, Christensen GD. Phenotypic selection of small colony variant forms of *Staphylococcus epidermidis* in the rat model of endocarditis. J Infect Dis 1988; **157**: 757–63.
8. Baddour LM, Christensen GD, Lowrance JH, Simpson WA. Pathogenesis of experimental endocarditis. Rev Infect Dis 1989; **11**: 452–63.
9. Baddour LM, Barker LP, Christensen GD, Parisi JT, Simpson WA. Phenotypic variation of *Staphylococcus epidermidis* in infection of transvenous endocardial pacemaker electrodes. J Clin Microbiol 1990; **28**: 676–9.
10. Christensen GD, Baddour LM, Hasty DL, Lowrance JH, Simpson WA., Microbial and foreign body factors in the pathogenesis of medical device infections. In: Bisno AL, Waldvogel FA, eds. Infections associated with indwelling medical devices. Washington: American Society for Microbiology, 1989; 27–59.
11. Deighton MA, Pearson SR, Capstick J, Spelman D, Borland R. Phenotypic variation of *Staphylococcus epidermidis* isolated from a patient with native valve endocarditis. J Clin Microbiol 1992; **30**: 2385–90.
12. Nash P, Krenz MM. Culture media. In: Balows A, Hausler WJ, Herrmann KL, Isenberg HD, Shadomy HJ, eds. Manual of clinical microbiology. Washington: American Society for Microbiology, 1991; 1226–314.
13. Schleifer KH. Gram-positive cocci. In: Sneath PHA, Mair NS, Sharpe ME, Holt JG, eds. Bergey's manual of systematic microbiology. Baltimore: Williams and Wilkins, 1986: 999–1103.
14. Kloos WE, Lambe DW. Staphylococcus. In: Balows A, Hausler WJ, Herrman KL, Isenberg HD, Shadomy HJ, eds. Manual of clinical microbiology. Washington: American Society for Microbiology, 1991: 222–37.
15. Christensen GD, Simpson WA, Bisno AL, Beachey EH. Adherence of slime-producing strains of *Staphylococcus epidermidis* to smooth surfaces. Infect Immun 1982; **37**: 318–26.
16. Deighton MA, Balkau B. Adherence measured by microtiter adherence as a virulence marker for *Staphylococcus epidermidis* infections. J Clin Microbiol 1990; **28**: 2442–7.
17. Christensen GD, Simpson WA, Younger JJ, et al. Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. J Clin Microbiol 1985; **22**: 996–1006.
18. Rosenberg M, Gutnick D, Rosenberg E. Adhesion of bacteria to hydrocarbons: a useful technique for studying cell surface hydrophobicity. FEMS Microbiol Lett 1980; **9**: 29–33.
19. Lyon BR, May JW, Skurry RA. Analysis of plasmids in nosocomial strains of multiple antibiotic-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother 1983; **23**: 817–26.
20. Crosa JH, Falkow S. Plasmids. In: Gerhardt P, Murray RGE, Costilow RN, et al., eds. Manual of methods in general bacteriology. Washington: American Society for Microbiology, 1981; 266–82.
21. Freeman DJ, Falkiner FR, Keane CT. New method for detecting slime production by coagulase-negative staphylococci. J Clin Pathol 1989; **42**: 872–4.
22. Gristina AG. Biomaterial-centered infection: microbial adhesion versus tissue integration. Science 1987; **237**: 1588–95.
23. Gemmell CG, Roberts E. Toxins and enzymes of coagulase-negative staphylococci isolated from human infections. J Hyg Epidemiol Microbiol Immunol 1974; **18**: 276–80.
24. Christensen GD, Baddour LM, Madison BM, et al. Colonial morphology of staphylococci on Memphis agar: phase variation of slime production, resistance to β-lactam antibiotics, and virulence. J Infect Dis 1990; **161**: 1153–69.
25. Finlay BB, Falkow S. Common themes in microbial pathogenicity. Microbiol Rev 1989; **53**: 210–30.
26. Borst P, Greaves DR. Programmed gene rearrangements altering gene expression. Science 1987; **235**: 658–67.
27. Woods DE, Sokol PA, Bryan LE, et al. *In vivo* regulation of virulence in *Pseudomonas aeruginosa* associated with genetic rearrangement. J Infect Dis 1991; **163**: 143–9.
28. Shapiro JA. Multicellular behavior of bacteria. ASM News 1991; **57**: 247–53.
29. Hall BG. Increased rates of advantageous mutations in response to environmental challenges. ASM News 1991; **57**: 82–6.