Interaction between *Pseudomonas aeruginosa* and *Staphylococcus aureus*: description of an anti-staphylococcal substance

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Summary. The presence of *Pseudomonas aeruginosa* in the sputum of 191 patients with cystic fibrosis was significantly related ($p < 0.0001$) to the absence of *Staphylococcus aureus*. Cross-streaking tests showed that 40 of 50 clinical strains of *P. aeruginosa* produced substances that inhibited the growth of *S. aureus*. When incorporated into agar plates, this antibacterial substance(s) inhibited the growth of 177 of 189 strains of nine staphylococcal species, all of 16 methicillin-resistant *S. aureus* and 27 of 39 strains of six other gram-positive genera. The substance(s) did not inhibit 23 strains of seven gram-negative genera tested. The antibacterial activity was heat stable and could be extracted into chloroform; activity was retained on Sephadex G-15 ($V/V_0 \approx 2$, $M_r < 500$) and eluted as a single peak from high performance liquid chromatography, well separated from pseudomonic acid, pyocyanin and a number of other phenazines.

Introduction

Staphylococcus aureus is usually the initial bacterial pathogen in the lungs of children with respiratory disease accompanying cystic fibrosis (CF); with increasing age, *Pseudomonas aeruginosa* begins to predominate.¹ Although antibiotic therapy is probably a major factor contributing to the decline of the staphylococci, bacterial antagonism may also play an important part in the establishment of *P. aeruginosa* in the lungs of these patients. The ability of *P. aeruginosa* to antagonise other bacteria was first demonstrated in 1899.^{2,3} Forty-two years later, Schoental⁴ prepared three antibacterial extracts of *P. aeruginosa*: the phenazine pigments, pyocyanin and 1-hydroxyphenazine, and an almost colourless substance derived from an old culture. Young⁵ confirmed the activity of these extracts but found that the antibiotic effect was absent from non-pigmented cultures. The report that biologically prepared 1-hydroxyphenazine has antibiotic activity⁴ was probably due to contamination by pyocyanin.^{6–8} Some studies have shown that *P. aeruginosa* produces substances antagonistic towards *S. aureus*. The mechanism of inhibition of staphylococcal growth may be due to nutrient depletion in mixed growth with

P. aeruginosa, the production of specific metabolic inhibitors, or to lytic enzyme(s).^{9–11} We have identified a low-molecular-weight hydrophobic antibacterial factor, distinct from pyocyanin and other phenazines, which is produced by clinical isolates of *P. aeruginosa* and which may be responsible for the anti-staphylococcal activity of *P. aeruginosa* in CF patients.

Materials and methods

Bacteria

Fifty clinical isolates of *P. aeruginosa*, mostly from CF patients, and 323 isolates of other species tested for sensitivity to the antibacterial activity of *P. aeruginosa* were obtained from cultures submitted to the Division of Hospital Infection, Central Public Health Laboratory, for epidemiological typing. Gram-positive bacteria included 261 staphylococci: *S. aureus* (99 strains, 16 of which were methicillin resistant), *S. capitis* (15), *S. epidermidis* (33), *S. haemolyticus* (19), *S. hominis* (16), *S. saprophyticus* (29), *S. simulans* (26), *S. warneri* (14), *S. xylosus* (10); and 39 strains of six other genera, *Micrococcus* spp. (5 strains), *Corynebacterium* spp. (6), *Bacillus* spp. (14), *Listeria* spp. (8), *Kurthia zopfii* (2), and faecal streptococci (4). Gram-negative bacteria tested included: *Acinetobacter* spp. (3 strains), *Enterobacter cloacae* (3), *Escherichia coli* (3), *Haemophilus influenzae* (4), *Klebsiella pneumoniae* (3), *Proteus* spp. (4) and *Serratia marcescens* (3). Other

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Pseudomonas spp. tested were also obtained from DHI and included: *P. fluorescens* (4 strains), *P. putida* (2), *P. cepacia* (2), *P. mendocina* (2) and *P. diminuta* (2).

Bacteria were grown on nutrient agar (Oxoid) and, when appropriate, on horse blood (7% v/v) agar or chocolate agar. The following media were used in addition: Kings "A" agar,¹² mannitol salt agar (MSA; Oxoid), *Pseudomonas* Isolation Agar (PIA; Difco), Cystine-Lactose Electrolyte Deficient Agar and Broth (CLED; Oxoid), Tryptone Soy Broth (TSB; Oxoid), and Todd-Hewitt Broth (Difco). Plates were incubated at 37°C for up to 48 h. Culture filtrates were prepared from broth cultures of *P. aeruginosa* which were centrifuged at 8000 *g* for 30 min and the supernates were filtered through a 0.45- μ m filter (Gelman Sciences Ltd, Northampton). Sputum obtained from 191 patients (aged 16 years or more) with CF, was digested by pancreatin (BDH, Poole, Dorset) at 37°C for 2 h¹³ and plated on selective and non-selective agar for the isolation of *P. aeruginosa* and *S. aureus*.

Antibacterial activity

Antibacterial activity produced by different strains of *P. aeruginosa* was demonstrated in four ways.

Cross-streak tests. An agar plate was seeded with a 4-h TSB culture of a strain of *P. aeruginosa* applied with a sterile cotton swab in a 1-cm wide streak. After incubation at 37°C for 24 h, the bacterial growth was removed from the plate with a microscope slide and the residual micro-organisms were killed by exposure to chloroform vapour for 30 min. TSB cultures of staphylococcal strains were diluted one in 100 in fresh broth and streaked with a loop at right angles to the line of the original inoculum. The plates were incubated at 37°C for 18 h and examined for inhibition of the indicator strain in the area that had supported the growth of the producer strain.

Well-plate assay. Lawns of the staphylococcal strain S1940 were prepared by flooding the surface of a Kings "A" agar plate with a 3-h broth culture. The excess fluid was removed and the surface of the agar was allowed to dry in air. Wells cut in the agar with a sterile metal punch (7 mm diameter) were filled with 200 μ l of filtrates of 48-h broth cultures of *P. aeruginosa* strain P3940 or fractions obtained by high performance liquid chromatography (HPLC). The plate was incubated at 37°C for 18 h and the zone of inhibition of growth of the staphylococcus was recorded.

Growth in mixed culture. Four-hour CLED broth cultures of *P. aeruginosa* strain P3940 or *S. aureus* strain S1940 were diluted with fresh broth to give equal optical density at 600 nm as measured on a nephelometer ($OD_{600} = 0.10$), and were then mixed in equal (5-ml) volumes. Controls of the same volume were prepared from sterile broth and also from broth cultures of each organism. During incubation at 37°C, 100- μ l samples were taken at 20-min intervals for 1 h, hourly intervals for 6 h, and again after 24 h. Viable counts were determined by a standard dilution

technique on MSA, PIA and Kings "A" agar media. To determine whether changes in viable count observed were due to pre-formed antibacterial factor, a filtrate (5 ml) of a 4-h *P. aeruginosa* broth culture was added to a 4-h *S. aureus* broth culture (5 ml). A chloroform extract of 10 Kings "A" agar plates on which *P. aeruginosa* P3940 had been cultured for 24 h was added to a 4-h *S. aureus* broth culture. A chloroform extract of 10 Kings "A" agar plates was used as a control. Viable counts were performed at similar intervals, as above.

Agar-plate incorporation. Nutrient agar plates were prepared containing either partially-purified factor (from Sep Pak®, see below), 20 μ g/ml loaded in 200 μ l of methanol, or an equivalent volume of methanol vehicle as a control. A 36-rod applicator was used to apply 0.3 μ l of diluted broth cultures of 244 gram-positive strains containing *c.* 10⁴ cfu/ml on to the plates which were then incubated at 37°C. Growth was recorded after overnight culture and after 1 week at room temperature.

Purification and characterisation of antibacterial factor(s) from *P. aeruginosa*

A non-pigmented clinical isolate of *P. aeruginosa* strain P3940 was grown on Kings "A" agar plates at 37°C for 24 h. The bacterial growth was removed and the agar was cut into small pieces, transferred to a conical flask and extracted with chloroform (three volumes of 500 ml each per 100 plates). The chloroform extract was loaded on to 10 silica Sep-Paks® (Waters Associates, Harrow, Middlesex) and the activity was eluted with methanol. HPLC was performed with a Waters Associates dual pump system on a μ Bondapak C₁₈ column (30 cm $\times$ 0.8 cm), eluting at 2 ml/min with a 25-min linear gradient of propan-2-ol in aqueous acetic acid 5% (20:80–40:60 v:v).¹⁴ Fractions (2 ml) were assayed by the well-plate technique after solvent removal under vacuum. The factor was also subjected to chromatography on Sephadex G-15 (32 $\times$ 1.5 cm; Pharmacia, Milton Keynes) eluting at 30 ml/h in methanol:ammonia (.880):water (2:1:2 v:v:v).¹⁵ Fractions (3 ml) were collected. Samples of the antibacterial factor were treated with either 2M HCl (60°C, 18 h), acetic anhydride in methanol (1:3 v:v, 1 h, 20°C) or methanolic hydrogen chloride (0.4 M, 1 h, 20°C).¹⁴ All solvents and reagents were removed under vacuum and resuspended in phosphate-buffered saline (PBS) for assay by the well plate or mixed culture method. Reagents blanks were inactive in the assay. Authentic pyocyanin and 1-hydroxyphenazine standards were synthesised as previously described.^{6, 8, 16}

Results

Samples of sputum from 191 patients with CF were examined and 184 were found to be infected with

P. aeruginosa, *S. aureus* or both. *P. aeruginosa* was present in the sputum of 146 of 191 patients, of which 67 (46%) were also colonised by *S. aureus*. Of the 45 patients who did not yield *P. aeruginosa* from their sputum, 38 (84%) were positive for *S. aureus*. There was a positive correlation between the presence of *P. aeruginosa* and the absence of *S. aureus* ($\chi^2=176.89$; $p<0.0001$), suggesting that suppression of *S. aureus* growth by *P. aeruginosa* may have occurred.

In an initial screen, 40 of 50 *P. aeruginosa* strains inhibited the growth of six randomly selected strains of *S. aureus* in the cross-streak assay and produced large (~30 mm) zones of inhibition. Four of the *P. aeruginosa* strains were weakly active, and the remaining six did not inhibit the growth of any of the staphylococci tested. Five strains of *P. aeruginosa* that exhibited strong anti-staphylococcal activity were tested for activity against 50 randomly selected strains of *S. aureus*. The growth of all staphylococci was suppressed by each of the *Pseudomonas* strains but the zones of inhibition varied with the agar medium used. Inhibition of staphylococcal growth was maximal on CLED and Kings "A" agar but was markedly reduced on blood agar. Further tests with other *Pseudomonas* spp. showed that anti-staphylococcal activity was particularly associated with *P. aeruginosa*: only two of four strains of *P. fluorescens* inhibited the growth of 10 strains of *S. aureus* (randomly selected from the original 50) whereas two strains each of *P. putida*, *P. cepacia*, *P. mendocina* and *P. diminuta* did not affect staphylococcal growth.

P. aeruginosa P3940, a non-pigmented strain with strong anti-staphylococcal activity, was incubated with sensitive strain *S. aureus* S1940 in broth culture. Within 20 min the viable count of the staphylococcus was reduced to below detectable levels, and that of the pseudomonas remained constant. Regrowth of strain S1940 did not occur after overnight incubation in the presence of *P. aeruginosa* (fig. 1). *P. aeruginosa* strain P3888, which did not inhibit the growth of *S. aureus* S1940 in preliminary cross-streak tests, did not reduce the viable count of strain S1940 after incubation in mixed culture for 6 h (fig. 1).

A 4-h culture filtrate of *P. aeruginosa* P3940 did not decrease the viability of *S. aureus*, whereas an 18-h culture filtrate from *P. aeruginosa* decreased the viability of *S. aureus* by one order of magnitude (c. 10-fold). The antibacterial activity present in the 18-h culture filtrate was completely removed by chloroform extraction and a much greater effect (10 000-fold) was observed when a chloroform extract of agar (after an 18-h growth of *P. aeruginosa* P3940 was removed) was incubated with *S. aureus* S1940 (fig. 2). These results suggest that this strain produces a lipid-like factor or factors that inhibit the growth of *S. aureus*. After overnight incubation, regrowth of the staphylococcus was observed; this could have resulted from exhaustion or inactivation of the bactericidal activity, as subcultures of *S. aureus* S1940 taken from the vessel at 6 h

Fig. 1. The effect of *P. aeruginosa* on the growth of *S. aureus* in mixed culture. Viable counts (cfu/ml) are shown for *S. aureus* S1940 in mixed culture (▲—▲) with *P. aeruginosa* P3940 (○—○), with controls showing the growth of *S. aureus* S1940 alone (△—△) and during co-incubation with a second strain of *P. aeruginosa* (P3888) which was inactive in the cross-streak test (□—□). The limit of detection of the assay was 10^2 cfu/ml.

remained sensitive to fresh extract of *P. aeruginosa* P3940.

The antibacterial activity was soluble in chloroform, but only poorly soluble in PBS or ether; it was not destroyed by boiling (30 min), freezing or mild acid hydrolysis, although a partial reduction in activity did occur after acetylation and esterification, suggesting the presence of amino, hydroxyl or carboxyl functions. Activity eluted as a single low molecular weight peak on Sephadex G15 ($V/V_0 \approx 2$, <500 Da); elution was undertaken with methanolic ammonia, a solvent system originally developed for the gel filtration of leukotriene D_4 ¹⁵ to overcome poor water solubility.

To obtain sufficient purified material for further study, *P. aeruginosa* P3940 was grown on 100 Kings "A" agar plates and activity was extracted with chloroform. Activity was retained on silica Sep-Paks® and could be eluted with methanol. Small amounts of the factor were purified further by reverse phase HPLC. The major peak of bioactivity eluted in fractions 33/34, well separated from pseudomonic acid (fraction 23/24) and the common pseudomonas phenazines—pyocyanin, 1-hydroxyphenazine, 1-carboxyphenazine and phenazine-1-carboxamide (fractions 9–14, fig. 3). The high percentage of organic modifier in the elution solvent indicated the hydrophobic nature of the material.

Using the Sep-Pak®-purified material, the activity of the antibacterial factor was tested by the agar plate assay against 244 gram-positive bacteria (table). At a concentration of 20 µg/ml, this material inhibited the growth, during incubation for 24 h, of 193 of 205 strains of staphylococci, including the 16 methicillin-resistant *S. aureus* (MRSA) strains and a high-level pseudomonic acid-resistant strain of *S. aureus* (MIC >1028 mg/L). When the plates were kept at room temperature for a further 7 days and re-examined: 57

Fig. 2. A chloroform extract of 10 Kings "A" agar plates on which *P. aeruginosa* P3940 had been cultured for 24 h caused a rapid reduction in the viability of *S. aureus* S1940 (●—●), which showed some recovery after 24 h. A chloroform extract of 10 non-inoculated Kings "A" agar plates alone had no effect (O—O). Viability of *S. aureus* S1940 is expressed as cfu/ml.

Fig. 3. HPLC profile of the antibacterial factor showing (a) A_{280} UV absorbance and (b) antibacterial activity ($\square$). Activity is expressed as the size of the zone of inhibition in the well-plate assay. Activity elutes in fractions 33/34, and is well resolved from pyocyanin and the phenazines (fractions 9–14) and pseudomonic acid (fraction 23/24).

of 189 strains produced macroscopic growth. Furthermore, 27 of 39 strains of the six other gram-positive genera tested were inhibited by the factor after incubation for 24 h: *Micrococcus* spp. (5 of 5), *Corynebacterium* spp. (6 of 6), *Bacillus* spp. (12 of 14), *Listeria* spp. (2 of 8), *Kurt. zopfii* (2 of 2) and faecal streptococci (0 of 4).

However, the antibacterial factor was inactive against 23 strains from seven gram-negative genera tested. In comparison, pyocyanin 32 $\mu\text{g/ml}$ had a wider spectrum of activity, inhibiting the growth of 50 of 56 strains of *S. aureus* and 14 of the same group of 23 gram-negative strains: *Acinetobacter* spp. (3 of 3), *Ent. cloacae* (3 of 3), *E. coli* (2 of 3), *H. influenzae* (4 of 4), *K. pneumoniae* (2 of 3), *Proteus* spp. (0 of 4), and

Table. Effect of partially-purified pseudomonas antibacterial factor (20 $\mu\text{g/ml}$) in nutrient-agar plates on the growth of staphylococci

Staphylococcus sp.	Number tested	Number of strains growing on agar plates at	
		24 h	7 days
<i>S. aureus</i>	27	0	0
<i>S. capitis</i>	15	0	6 (2)
<i>S. epidermidis</i>	33	0	6 (4)
<i>S. haemolyticus</i>	19	1 (1)	5 (3)
<i>S. hominis</i>	16	1 (1)	4 (4)
<i>S. saprophyticus</i>	29	5 (5)	20 (11)
<i>S. simulans</i>	26	1 (1)	3 (2)
<i>S. warneri</i>	14	4 (4)	7 (6)
<i>S. xylosus</i>	10	0	6
MRSA	16	0	...
Total	205	12 (12)	57 (32)

(n), Number of bacteria that exhibited poor growth on agar incorporating the factor.

S. marcescens (0 of 3). 1-Hydroxyphenazine 32 $\mu\text{g/ml}$ was inactive against all strains tested.

Discussion

Colonisation of the respiratory tract by *P. aeruginosa* may be aided, in part, by the production of antibacterial factors active against the resident commensal flora and other pathogenic bacteria. The apparent reduction in staphylococcal colonisation of adult CF patients consequent to pseudomonas infection prompted us to investigate the production of an antibacterial factor expressed by *P. aeruginosa*.

There have been several previous reports identifying antimicrobial factors from *P. aeruginosa*^{5, 17, 18} but these substances cannot account for the activity of *P. aeruginosa* described here. Pseudomonic acid and the phenazines have a different spectrum of antibacterial activity and are readily separated from the antibacterial factor by HPLC. The substance reported by Zyskind *et al.*¹⁹ which inhibited *S. aureus* was of high molecular weight. The action of the factor described in the present paper is rapid on *S. aureus*, both in mixed culture and when added to broth, in contrast to that found by Perestelo *et al.*²⁰ who reported an anti-staphylococcal effect of *P. aeruginosa* only after incubation for 16 h. The microcins originally described as a new family of antibiotics produced by *E. coli*²¹ are now known to be distinct chemical classes of antibiotics, with markedly variable structure and molecular weight.^{22, 23} In the initial report,²¹ evidence was adduced that two strains of *P. aeruginosa* also produced microcins with antibacterial activity, although both had some activity against *E. coli* and are thus distinct from the factor reported in our study.

The anti-staphylococcal activity produced by

P. aeruginosa may explain the high correlation between the absence of *S. aureus* in the presence of *P. aeruginosa* in CF, and the association of *P. aeruginosa* colonisation in CF patients with a decline in staphylococcal infections. We have purified this antibacterial factor, and shown that it is a low molecular weight hydrophobic species. Because of these properties, it may escape neutralisation by the host defence system *in vivo* and be a further factor contributing to the success of pseudomonas colonisa-

tion and establishment of *P. aeruginosa* in the lungs of patients with CF. The activity against a number of staphylococci, including MRSA strains and at least one high-level pseudomonic acid-resistant strain, raises the possibility that this substance may have some clinical application.

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