Transformation with Bacteriocin Factors in Staphylococci

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SUMMARY

Transfer of bacteriocin factors from bacteriocinogenic to other staphylococci was observed at high frequency on the basis of paradoxical inhibition (p.i.) on two media (BTM and MPI). Colonies of non-bacteriocinogenic sensitive bacteria from the central growth of p.i. showed acquisition of bacteriocinogeny and bacteriocin resistance at high frequencies which was not lost in the course of 18 h. Acquired bacteriocinogeny was a stable character and had an overall frequency of 10^{-2} to 10^{-3} among different recipient populations. Conjugation, transduction and transfection processes could be excluded, and the gene transfer process was considered to be transformation with plasmid DNA.

INTRODUCTION

A central region of growth has been observed within the zone of inhibition produced by bacteriocinogenic bacteria among *Escherichia coli* (Fredericq, 1948), *Shigella sonnei* (Abbott & Shannon, 1958), *Pseudomonas aeruginosa* (Wahba, 1963), *Vibrio cholerae* (Chakrabarty, Adhya, Basu & Dastidar, 1970) and among different members of *Shigella*, *Salmonella*, *Escherichia*, *Proteus*, *Vibrio* and *Alcaligenes* spp. (Chakrabarty & Dastidar, 1974). Chakrabarty, Adhya, Basu & Dastidar (1970) called the phenomenon paradoxical inhibition (p.i.) and Chakrabarty & Dastidar (1974) demonstrated that transfer of bacteriocinogeny from the primary to the secondary bacteria which constituted the central growth took place at high frequency through a process of plasmid-mediated genetic transformation. Bacteriocinogeny is also known among staphylococci (Parker, Tomlinson & Williams, 1955; Barrow, 1963; Fink & Ortel, 1969; Gagliano & Hinsdill, 1970; Jetten, Vogels & de Windt, 1972). We report on transfer of bacteriocinogeny among staphylococci observed in the p.i. system.

METHODS

Bacteria. The strains used are described in Table 1. The stock cultures were maintained as described by Chakrabarty & Dastidar (1974).

Tests for p.i. and transformation. All the media and methods used are essentially those of Chakrabarty & Dastidar (1974); only the modifications are described here. Bacteriocinogeny was studied on (i) Tryptone soya broth (Oxoid, CM129) 2 %, agar (Oxoid, No. 3, L₁₃) 1.2 %, plus 5 % sterile defibrinated sheep blood, and (ii) the bacteriocin typing medium (BTM, see

Table 1. *Cultures*

Description*	Group†	Subgroup†	No. of strains	Source
<i>Staphylococcus aureus</i> 6571 ‡(+), 8530 ‡, 8531 ‡, 8532 ‡	I	I	4	S. P. Lapage, London
<i>S. aureus</i> SA1, SA8(+), SA12(+), SA22	I	I	4	Choudhuri & Chakrabarty (1970)
<i>S. aureus</i> B8, B9, B43, B45(+)	I	I	4	N. Gupta, Calcutta, India
<i>S. aureus</i> ML32, ML34, ML42, ML62, etc.	I	I	4, 7(+), 13	M. R. Lahiri, Calcutta, India
<i>S. aureus</i> others	I	I	11(+), 15	Authors
<i>S. saprophyticus</i> 28	I	II	1	N. W. Preston, Manchester
<i>S. saprophyticus</i> WS2, WS9, VS14, etc.	I	II	3, 11	Choudhuri & Chakrabarty (1970)
<i>S. saprophyticus</i> B2, B4, B12, B24	I	II	4	N. Gupta, Calcutta, India
<i>S. saprophyticus</i> ML6(+), ML12, ML47, ML123(+)	I	II	4	M. R. Lahiri, Calcutta, India
<i>S. saprophyticus</i> , etc.	I	II	8(+), 10	Authors
<i>Staphylococcus</i> spp.	I	IV-VI	10	M. R. Lahiri, Calcutta, India
<i>S. aerfermentans</i> 60	2	7	1	N. W. Preston, Manchester
	(<i>Micrococcus</i>)			
<i>S. aerfermentans</i> , etc.	2	7	3	Authors

(+), Nonbacteriocinogenic strains.

* According to Shaw, Stitt & Cowan (1951).

† According to Baird-Parker (1965).

‡ Numbers of National Collection of Type Cultures, London.

Chakrabarty *et al.* 1970) consisting of the above medium with 0.05 % tri-sodium citrate. The medium for paradoxical inhibition (MPI) for *Staphylococcus* was casein hydrolysate-yeast extract agar with 0.008 % sodium dodecyl sulphate (SDS) and 0.001 % EDTA, pH 7.4 to 7.5. In the basic technique for demonstration of bacteriocinogeny and p.i., incubation of primary bacteria was for 72 h and overnight refrigeration of BTM plates was omitted. For producing a continuous band of central growth (as in acquisition and frequency tests, see below) 0.1 ml of a 1:10 dilution of the culture was spread uniformly over the surface of the medium. In assay of bacteriocins in the extracted fluids, the method of Gagliano & Hinsdill (1970) was followed. In the tests for acquisition of bacteriocinogeny, growth samples of different portions of the secondary inoculum were obtained 6 and 18 h after application. In tests for p.i. on a cell-free surface, both BTM and MPI media were used, and other procedures remained unchanged. The tests for extracellular mediator of p.i. were modified by lowering the final concentration of SDS to 0.001 % in the shake culture fluid, keeping other procedures the same. In 'curing' tests, SDS was tested within the range 0.005 to 0.03 % at intervals of 0.005 %, for determination of its minimum inhibitory concentration and 'curing' effects on different strains of staphylococci.

RESULTS

Bacteriocinogeny among staphylococci on BTM

Preliminary studies on bacteriocinogeny among staphylococci were made on 64 strains of group I, comprising 30 *Staphylococcus aureus* (subgroup I), 24 *S. saprophyticus* (subgroup II) and ten other strains (from other subgroups) (Baird-Parker, 1965). These strains were

Fig. 1. The inhibition pattern of bacteriocinogenic strain *Staphylococcus aureus* ML34 on BTM. The indicator bacteria, in the order 1 to 8, are: *S. saprophyticus* vs14 and ML6, *S. aureus* B43, SA22 and B45, *S. saprophyticus* ML123 and ws2, and *S. aureus* SA1. Numbers 1, 7 and 8 show inhibition (+++, up to 9 mm); 3 and 5 are resistant (—), while 4 shows markedly reduced growth over the central area (±). Two plaque-like clearings on the left peripheral growth of no. 5 suggest phage action, and appear to have been derived from the indicator bacterium itself as its streaking was started from the left. Numbers 2 and 6 show p.i. with all the three morphological components, but differ from each other with respect to the width of their inhibition zones (+++ or > 9 mm, and ++ or up to 6 mm, respectively), the density of the central growths (more in no. 6) and their symmetry on two sides of the midline (fusion is complete in 2 but incomplete in the upper portion of 6).

divided into eight batches, members of which were tested against each other as primary and secondary bacteria; 21 producer and 29 indicator bacteria were thereby detected. Eight of the indicator strains were finally selected on the basis of maximum distinguishing capacity and stability of their reactions. Incidence of bacteriocinogeny among 117 strains of staphylococci (Table 1) was 38 (32.5%) on BTM without citrate and 85 (72.7%) on complete BTM, as detected by the indicator set. The reactions of different indicator bacteria are shown in Fig. 1.

Paradoxical inhibition among staphylococci on BTM and MPI

Paradoxical inhibition was commonly encountered on BTM without citrate. The incidence of p.i. with eight indicator staphylococci tested against 85 bacteriocinogenic strains (Table 1) used as primary strains on complete BTM was 39, 33, 24, 18, 11, 3, 0 and 0. Studies on the effects of different combinations of SDS, EDTA, tri-sodium citrate and pH in various concentrations in casein hydrolysate-yeast extract agar medium on p.i. showed that SDS at concentrations of 0.005 to 0.015% and pH 7.2 to 7.6 promoted p.i. EDTA alone was ineffective, but at concentrations between 0.0005 and 0.0015% it enhanced the effects of SDS. Tri-sodium citrate did not have any effects. When ten different bacteriocinogenic

Fig. 2. Paradoxical inhibition produced by *Staphylococcus aureus* ML34 on MPI medium. The secondary bacteria, in the order 1 to 5, are: *S. saprophyticus* ML123, *S. aureus* SA1 and SA8, and *S. saprophyticus* vs14 and ws2. Only central growths, of different widths, are present.

staphylococci were tested against these indicators on MPI, p.i. was observed between all the reacting pairs in at least one of the three incubation schedules of the primary inocula, e.g. 54, 64 and 72 h. Certain indicators, e.g. *Staphylococcus saprophyticus* WS2, ML6 and ML123, exhibited higher frequency of p.i. compared with others (cf. incidence of p.i. on BTM). The frequency of p.i. between representative staphylococcus and micrococcus strains belonging to different subgroups of Baird-Parker (1965), when tested against each other on MPI, was found to be very high. Only bacteriocinogenic bacteria used as primary inocula on MPI produced p.i. with its own, other bacteriocinogenic and non-bacteriocinogenic secondary inocula. When the primary and secondary inocula belonged to different cultures, central growths of p.i. identified as described in Table 1 were found to belong to the secondary inocula. Exposure to chloroform vapour for 2 h killed all the staphylococcal cultures examined: even after prolonged incubation, tests in which secondary inoculations were omitted or done as parallel streaks leaving areas of uninoculated medium did not yield growth either in the medium or within the uninoculated zones.

Morphology of p.i. on BTM and MPI. These were as described elsewhere (Chakrabarty & Dastidar, 1974; see also Figs. 1 and 2).

Assay of bacteriocins

The bacteriocin titres on BTM were highest in the central zone, followed by the zones of inhibition; no bacteriocin activity could be detected in the peripheral zones. On MPI, in contrast, the titres in the different zones were nearly equal. Replicate plates used as controls showed p.i. with the indicator strains used for assay.

Table 2. *Bacteriocin inhibition patterns of recipient colonies of Staphylococcus aureus SA8*

Colonies from central growth of p.i., selected at random but in approximately equal numbers from BTM and MPI, were tested for bacteriocinogeny as primary bacteria. Forty of 47 colonies acquired bacteriocinogeny.

Indicators	Inhibition patterns					
	Primary <i>S. aureus</i> ML34	Secondary <i>S. aureus</i> SA8	Recipient colonies (SA8)			
			1	2	3	4
<i>S. saprophyticus</i> VS14	+++	—	+++	—	—	—
<i>S. saprophyticus</i> ML6	PI++++	—	PI++	+++	+++	PI++
<i>S. aureus</i> B43	—	—	—	—	—	—
<i>S. aureus</i> SA22	±	—	—	—	—	—
<i>S. aureus</i> B45	—	—	—	—	—	—
<i>S. saprophyticus</i> ML123	PI++	—	PI++	—	—	PI++
<i>S. saprophyticus</i> WS2	+++	—	+++	+++	—	+++
<i>S. aureus</i> SA1	+++	—	+	—	—	—
No. of colonies of each type	(SC)	(SC)	16	13	7	4

+, ++, +++, +++++, Width of zones of inhibition, up to 3, 6, 9 and > 9 mm respectively; ±, markedly reduced growth in the centre; —, resistant; PI++, PI+++ and PI++++, width of central growth 4 mm and zones of inhibition up to 6 and 9 and > 9 mm, respectively; SC, stock culture.

Acquisition of bacteriocinogeny

Paradoxical inhibition occurred when the non-bacteriocinogenic strain *S. aureus* SA8 was inoculated as secondary culture against the bacteriocinogenic strain *S. aureus* ML34. The results showed that only colonies originating from the central growth of p.i. on BTM and MPI acquired bacteriocinogeny, while those belonging to other areas did not. The frequency of acquisition was high in all the tests and did not decline in the course of 18 h. Bacteriocinogeny, once acquired, was remarkably stable, as evidenced by tests repeated at intervals for a year; in addition, these colonies could produce p.i. as primary bacteria with members of the indicator set. The inhibitory patterns of some randomly selected bacteriocinogenic colonies are given in Table 2. Additional studies with *S. aureus* ML34 and ML32 as primary and *S. saprophyticus* ML6 and *S. aureus* SA12 as secondary bacteria respectively produced similar results; of 40 colonies of *S. saprophyticus* ML6 tested, 36 had acquired bacteriocinogeny and were distributed among four different inhibition patterns in the proportions 36, 28, 22 and 14%; similarly, of 42 colonies of *S. aureus* SA12 tested, 39 were bacteriocinogenic and distributed into four different patterns at 44, 26, 20 and 10%. Control experiments, with *S. aureus* SA8 and *S. saprophyticus* ML6 as primary bacteria against these same strains as secondary bacteria on BTM and MPI, failed to reveal acquired bacteriocinogeny.

Frequency of acquisition of bacteriocinogeny. Using *S. aureus* ML34 as primary inoculum, the frequencies for *S. aureus* SA8 and *S. saprophyticus* ML6 (secondary bacteria) on MPI were 2.2×10^{-2} and 1.6×10^{-3} respectively.

Paradoxical inhibition on cell-free surface

Primary bacteria were *Staphylococcus aureus* ML32 and ML34; secondary bacteria were *S. aureus* SA12 and SA8, and *S. saprophyticus* ML6, WS2 and ML123. Inoculations of secondary bacteria on reverse and agar overlaid surfaces on BTM and MPI produced p.i. similar to that seen on the control plates where the primary and secondary bacteria were on the same surface. Colonies from the central area of growth of p.i. belonging to *S. aureus* SA12 and SA8 against *S. aureus* ML32 and ML34 respectively showed acquisition of bacteriocinogeny.

Paradoxical inhibition with DNA preparation

The inclusion of DNA prepared from the bacteriocinogenic strain *S. aureus* ML34 in gutters and inoculation of secondary bacteria as in the preceding test, using the basic technique described in Methods, similarly produced p.i. The colonies derived from the central growths of p.i. of *S. aureus* SA12 and SA8 (non-bacteriocinogenic) against *S. aureus* ML34 showed acquired bacteriocinogeny. None of the non-bacteriocinogenic indicator bacteria or their DNA preparations produced p.i., nor did media containing no DNA preparations. DNase treatment of the DNA preparations abolished p.i. for all the indicator bacteria, while RNase treatment did not.

Effects of SDS on growth and bacteriocinogeny

Of 15 different staphylococcal strains tested, the number of strains that grew on 0.005, 0.01, 0.015, 0.02, 0.025 and 0.03 % SDS in liquid and solid media respectively were 15, 13, 7, 4, 1 and 0, and 13, 10, 5, 1, 0 and 0. The results showed that the 'curing' doses of SDS (0.005 to 0.015 %) were lower than the inhibitory ones and could entirely or partly 'cure' these cultures of bacteriocinogeny.

The behaviour of light (secondary) and heavy (primary) inocula on media containing different combinations of SDS and bacteriocins were studied with respect to five bacteriocinogenic strains. On BTM containing homologous bacteriocin, secondary light and heavy inoculations of these bacteria yielded good growth, except in one instance where p.i. occurred. Fresh MPI gave similar results for all the bacteria tested. However, in the presence of pre-formed bacteriocins resulting from a heavy primary inoculum, secondary heavy inocula of the homologous bacteria showed markedly reduced growth except over the central area where it was luxuriant, while secondary light inocula of homologous and heterologous bacteria produced p.i. Light primary inocula did not inhibit any of these cultures, irrespective of the type of secondary inoculum.

DISCUSSION

Two sorts of bacteriocin resistance were seen in the secondary inocula on plates: (i) that due to resistant colonies situated within the zones of inhibition (occurring in small numbers on BTM and rarely on MPI); as these colonies did not acquire bacteriocinogeny, they were considered to be mutants; (ii) that due to the central growth. The colonies constituting the latter (on BTM and MPI) grew in the presence of comparatively high concentration of bacteriocin and showed both bacteriocin 'resistance' and acquired bacteriocinogeny. Paradoxical inhibition and acquisition of bacteriocinogeny by these colonies depended on the presence of bacteriocins, on the primary culture being bacteriocinogenic, or on the free DNA from such cultures (as these were sensitive to DNase and resistant to RNase), but did not depend on cell contact. As in the previous study (Chakrabarty & Dastidar, 1974), the transfer process described here appears to be genetic transformation and the central growth appears to be due to transformant selection *in situ* by the bacteriocins.

The inhibition patterns of the recipient colonies of *S. aureus* SA8 belonged to four different types, only one of which (inhibition pattern 1) resembled that of the primary, *S. aureus* ML34 (Table 2). These findings suggest that the minimum number of transferred bacteriocin factors could be two, and transfer of such factors in different combinations probably occurs among staphylococci (cf. Chakrabarty & Dastidar, 1974). This coupled with a host-modified phenotypic expression (the inhibition pattern 1 of the transformant, *S. aureus* SA8, appeared

to be weaker than that of the donor) may account for different bacteriocin types among the transformants.

An evaluation of the role of tri-sodium citrate suggests that although it could stimulate bacteriocin production and protect DNA liberated in the medium (see Results and Hotchkiss, 1957), it is obligatory for neither bacteriocinogeny nor for p.i., and therefore does not wholly account for p.i. and transformation on BTM. The role of SDS in such processes, and in the mechanism of release of transforming DNA, had been discussed under similar situations (Chakrabarty & Dastidar, 1974). SDS at the concentration (0.008 %) used in MPI is unlikely to release DNA by lysing primary bacteria, but it could probably do so (i) by increasing the spontaneous breakdown of bacteria (Elek, 1959) and (ii) by releasing intracellular macromolecules as a result of its selective action on the cell membrane (Sonstein & Baldwin, 1972; Woldringh & van Itersen, 1972). Its 'plasmid curing' role also could be of significance. Our studies on SDS showed that it could eliminate the determinants of bacteriocinogeny from different wild and transformant (recipient) cultures at high frequency. The studies of Gagliano & Hinsdill (1969) also suggest that the bacteriocin factors among staphylococci are plasmid-linked and could be 'cured'. As in the earlier studies, the central growth of p.i. described here could be due to acquisition by cells of the secondary inoculum through the transformation process of plasmid DNA of the donor, released either spontaneously or as a result of the 'curing' effects of SDS.

It is evident that the transfer of bacteriocin factors among staphylococci is like that observed among enterobacteria, *Vibrio*, and *Alcaligenes* spp. (Chakrabarty & Dastidar, 1974). However, the acquisition process among the former appears to be more stable on MPI in comparison with that for other bacteria studied (Chakrabarty & Dastidar, 1974) because the acquired bacteriocin factors were substantially retained for 18 h or more. The causes for such a difference are not understood.

Genetic transformation among staphylococci was suggested by the works of Pakula, Hulanicka & Waczak (1958), Imshenetskii, Perova, Zaitseva & Belozerskii (1959), Lambina (1961), Komarov (1962), Dobrzanski, Osowiecki & Jagielski (1968), Smirnova, Gubenko & Rybakova (1969) and Nomura *et al.* (1971), although Elek (1959) and Spizzizen, Reilly & Evans (1966) considered this to be improbable. Lindberg & Novick (1973) demonstrated transformation with plasmid DNA in one strain of staphylococcus. Our present and earlier investigations (Chakrabarty & Dastidar, 1974) show that plasmid-mediated genetic transformation is a widely prevalent phenomenon among staphylococci and different groups of Gram-negative bacteria. Further studies may throw more light on this potentially important aspect of bacterial genetics.

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