FEMS Microbiology Letters 3 (1978) 323–326

Published by Elsevier/North-Holland Biomedical Press

ISOLATION AND GENETIC CHARACTERIZATION OF POLYMYXIN-RESISTANT MUTANTS OF *SALMONELLA*

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Received 28 March 1978

1. Introduction

Polymyxins are closely related membrane-active antibiotics which act preferentially on Gram-negative bacteria [1,2]. Their amphiphilic molecule binds strongly to various lipids [3], including those of the outer membrane of enteric bacteria. Polymyxins cause visible alterations apparently involving the outer membrane of the bacterial cell wall [4,5]. These facts and the resistance of Gram-positive bacteria compared with the sensitivity of Gram-negatives suggested that the outer membrane, peculiar to Gram-negative bacteria, might have an essential role in polymyxin action and, conversely, that polymyxin could be used as a probe of outer membrane (OM) properties.

In this hope we have started an analysis of polymyxin-resistant (PMR) mutants of *Salmonella*. We describe here their basic characterization and genetic determination. Their altered morphological response to polymyxin has been described [6,7], and their biochemical properties will be reported shortly (Vaara et al., manuscripts in preparation).

2. Material and Methods

The bacteria were *Salmonella typhimurium* and *S. abony* derivatives (Table 1). Mutants were isolated after treatment of cultures with diethylsulfate (dES) [8] or in some cases with *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (NG) [9], as indicated in Table 1. Genetic analysis was by conjugation [10] using several Hfr donors (Fig. 1).

Polymyxin (PM) was incorporated in nutrient agar plates at concentrations of 0.1, 0.3, 1, 3, 10, 30, 100, 300, and 1000 I.U. of Colistin E sulfate per ml; the plates were used within a few days of preparation since older plates gave inconsistent results. For isolation of PMR mutants, broth cultures were diluted 1 : 10 before spreading 0.1 ml of the dilution per plate (to give an inoculum of approx. $2 \cdot 10^7$ bacteria per plate), because too high an inoculum would absorb so much PM that its effective concentration would be reduced in a not easily controllable way. PM sensitivity of strains was, for the same reason, scored as growth of small inocula on these plates.

Sensitivity of the strains to several other antimicrobial agents was tested similarly by small inocula on nutrient agar containing one of the following: anionic detergents sodium deoxycholate (DOC, 0.4% at pH 8.3, fresh plates only), sodium cholate (0.3 to 3%) or sodium dodecyl sulfate (SDS, 0.1 to 1% at pH 7.0); the cationic detergent cetyl pyridinium chloride (100–500 μg/ml), the neutral detergent Triton X-100 (TX, 2%) gentian violet (10 μg/ml), or erythromycin (4.5 μg/ml).

3. Results and Discussion

3.1. Mutant isolation and characterization

PMR mutants were isolated both from smooth and rough parents of *S. abony* and *S. typhimurium* (Table 1). When the mutant gene was transferred by conjugation into the standard *S. typhimurium* rough (*rfaJ*) strain SH5014 the phenotype in respect of polymyxin resistance was the same, unaffected by strain.

The parent PM-sensitive (PMS) strains grew on nutrient agar containing 1–3 I.U. of PM/ml. Resistant

TABLE 1

Salmonella strains used

PMS [a] parent strains			Their PMR derivatives	Derivation [b]	PM phenotype
SA464 [c]	tm [d]	Hfr K1-2 *serA13 rfa-3058*	SH5619-24	dES	PMR30
SA534 [c]	tm	HfrK4 *serA13 rfa-3058*			
SH474 [c]	ab	Hfr H10 *thi-51*	SH5251-60	dES	PMR30
			SH5507	dES from SH5253	PMR300
SH33 [c]	ab	Hfr H7	SH5333-45	dES	PMR30
			SH5397-5411	dES from SH5339	PMR300
SH4247	tm	SD14 [e] *ilv-1178 thr-914 his-6116*	SH4413-30	NG	PMR30
SH5014	tm	*rfaJ4041* mutant of SH4247	SH5357, etc.	cross with SH5333	PMR30
			SH5585-6	dES from SH5357	PMR300
SH6482	tm	*ilv*$^+$*metE*$^+$ derivative of SH5014	SH6497-6501	dES	PMR30
			SH6550-51	dES from SH6497	PMR300
SW1444 [c]	ab	Hfr H1 *met-1151 aro-851 rpsL501*			

[a] PMS, polymyxin sensitive (grows on nutrient agar plates containing 1–3 μg of polymyxin E/ml). PMR, resistant to 30 (PMR30) or 100–300 (PMR300) μg/ml.

[b] Derivation either as mutant after treatment with diethylsulfate (dES) or nitrosoguanidine (NG), or as recombinant from conjugational crosses.

[c] (ref. 10).

[d] tm, *S. typhimurium;* ab, *S. abony.*

[e] Subline SD14 as described [15], *metA22 met E551 trpB2 xyl-404 rpsL120 flaA66*

mutants were isolated at frequencies of approx. 10^{-6} after mutagen treatment on plates containing 30 I.U. of PM/ml. They behaved like true mutants, growing on 30 but not 100 μg PM/ml (phenotype PMR30) unlike the reversible phenotypic variants selected by PM treatment in *Pseudomonas aeruginosa* [11]. However, on storage PMS cells appeared in the cultures – i.e. after 1 month, one clone out of 75, and after 1 year 27 out of 75 had turned PMS. Two possibilities for this instability were considered: form variation [12] or selective pressure favoring the PMS forms. Form variation seems unlikely because the PM-sensitive variants were stable: none of 40 clones tested after one year in storage had changed to PMR, and new PMR forms could be selected from these at the same low frequency as from the original parent. The PMS forms could on the other hand easily be thought to have a selective advantage since many PMR strains showed signs of membrane damage (see below).

Selection for mutants with a higher degree of resistance yielded such mutants at approx. 10^{-6} frequency from all the PMR30 mutants tested but not from PMS strains. The resistance was increased 3- to 10-fold, to PMR100 or PMR300.

Many PMR mutants – approx. 80% of those of *S. abony* and 50% of those of *S. typhimurium* – had become sensitive to a 0.4% concentration of DOC. The same strains had also a slightly increased sensitivity to sodium cholate, SDS, and cetyl pyridinium chloride but not to Triton X-100, gentian violet or erythromycin. The DOC-sensitive phenotype could be used to select further DOC-resistant derivatives of the PMR mutants. Each of eight PMR mutants tested gave such DOC-resistant "revertants", and in most cases (221 out of 230) these had become as sensitive to PM as the original parent strain. In one case studied the mutation to PMS was found to have occurred in the same area as the original PMR mutation.

3.2. Genetic determination of the PMR30 phenotype

Conjugational crosses with several different Hfr donors, injecting their chromosome clockwise or counterclockwise from separate points of origin, as shown in Fig. 1, showed that the mutation in each case had occurred in the same chromosomal area, close to position 0 or 100 of the proposed 100 unit *Salmonella* map (Sanderson and Hartman, manuscript

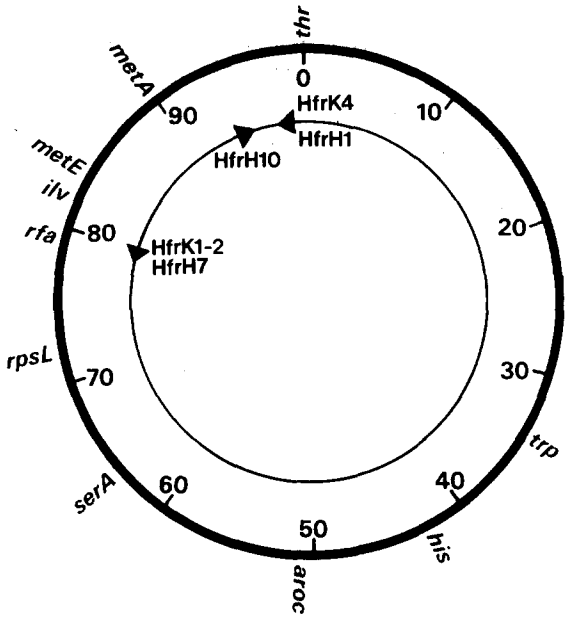

Fig. 1. A simplified map of *Salmonella* [10] (Sanderson and Hartman, manuscript in preparation) showing the direction and points of origin of chromosome injection by the Hfr strains used [10].

in preparation). We tentatively call this area *pmrA* although it may turn out to contain more than one cistron.

(1) When 12 separate PMR mutants of *S. typhimurium* were crossed with PMS HfrK1-2 donor, 53 to 80% of the recombinants with the donor *thr* or *metA-metE* alleles had become PMS like the donor, placing *pmrA* in the map area of 83 to 100. The same result was obtained when PMS sensitive recipients were crossed with each one of 13 separate PMR mutants of the donors HfrK1-2 or HfrH7.

(2) 11 PMR30 donors of HfrH10, which inject the chromosome counterclockwise starting at the approximate position of 90, could not transmit the PMR30 phenotype to recombinants selected for the donor allele of *metA* or *ilv*, excluding the map area 83 to 90.

(3) When PMS donors HfrH1 and HfrK4, both injecting their chromosome clockwise, starting very close to position 100, were crossed with *pmrA* mutants, 17% of recombinants with the donor allele of *thr*, and much fewer (0 to 11%) of those with the donor allele of *trp*, had become PMS. Thus the most likely position of *pmrA* would be close to *thr*, between it and the point of origin of these Hfr strains.

4. Discussion

We thus found the single step polymyxin-resistant mutants of *Salmonella* to be a genetically homogeneous group, defining a gene locus *pmrA* close to map position 100. The degree of PM resistance given by the *pmrA* mutations was an approx. 30-fold rise in resistance when determined for growth of small inocula on nutrient agar plates. Increased sensitivity of a majority of these mutants to deoxycholate suggested an alteration of the outer membrane. Polymyxin-resistant mutants have also been isolated in *E. coli* [13, 14], and share these two phenotypic properties. The molecular basis of the mutation was, however, not elucidated, nor did these studies give any data on the genes involved.

From the PMR30 mutants, we could isolate second step mutants to PMR100 or PMR300. The new mutation had occurred at a site separate and distant from *pmrA*, but so far we have not been able to locate it in a positive way (unpublished data).

Acknowledgements

We thank Ms. Sirkka Vene, Ms. Seijasisko Suurnäkki and Ms. Hannele Lehtonen for excellent technical assistance, and the Sigrid Jusélius Foundation and the Finnish Medical Research Council for grants to support this work.

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