

HIGH FREQUENCY OF AUXOTROPHY IN CLINICAL ISOLATES OF *Proteus mirabilis* HARBORING AN R PLASMID

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ABSTRACT

We report a novel phenomenon of high genetic instability, related to auxotrophy, in strains of *Proteus mirabilis*. Among *P. mirabilis* strains harboring the R plasmid kept in our laboratory collection, and some freshly isolated strains from clinical material, 54% of the samples presented auxotrophy at frequencies higher than 10^{-3} . Prototrophic clones gave rise to auxotrophic ones at frequencies not explainable by the usual mutation mechanisms. The instability mainly affected the carbamoyl phosphate synthetase gene (*car*), which leads to a double requirement for arginine and uracil for growth in minimal medium. Other genes were also affected, at a lower frequency. The *car* mutation does not revert to prototrophy. A similar phenomenon of instability was induced in *Escherichia coli* strain HB 101 upon introduction of a drug-resistance plasmid from *P. mirabilis*. We have ruled out the hypothesis of a transposon in the generation of auxotrophy.

INTRODUCTION

Proteus mirabilis is a rod-shaped aerobic, highly motile, enterobacterium, found in the soil, water, sewage as well as in animals and humans. It is found with some frequency in normal faeces, but often in greatly increased numbers in individuals receiving antibiotic therapy, or associated with diarrheal diseases due to other organisms. *P. mirabilis* is frequently the cause of urinary tract and other serious infections (Sonnenwirth, 1980). Its economic and ecological significance, the state of art for increasing yields of extracellular enzymes and the significance of advances in microbial genetics, molecular biology and biotechnology have been reviewed (Coetzee, 1972; Ramaley, 1979; Taylor and Richardson, 1979; Workman *et al.*, 1986; Costa, 1990).

Except when under the influence of extra-chromosomal elements, *P. mirabilis* is a genetically stable bacterium. It has been known for a long time that drug resistance plasmids (R plasmid) suffer rearrangements in *P. mirabilis*. Some R plasmids dissociate into transfer factor (RTF) and resistance determinants (r-det), which

then replicate under relaxed control (Falkow *et al.*, 1969; Cohen and Miller, 1970; Coetzee, 1972). The recombination process involves *ISI* copies present in the plasmid (Ptashne and Cohen, 1975). Plasmid associated drug-resistance markers have also been shown to be highly unstable in this species (Newton, 1986).

P. mirabilis secretes a single protease (gelatinase). Some strains exhibit a quite unstable protease phenotype with a loss of protease production (P^- colonies) of up to 10% of the progeny clones. Reversion of the protease-minus phenotype was never observed. Introduction of R-plasmids into protease-producing cells (stable ones) reduced the amount of protease-activity to about 50%, suggesting interference with expression or secretion of the protease (Costa *et al.*, 1985; Costa, 1990).

The present work deals with the high frequency of auxotrophy observed in clinical isolates of *P. mirabilis* harboring the R plasmid.

MATERIAL AND METHODS

Bacterial strains

The strains used in this work are described in Table I. *P. mirabilis* strains were kept on semi-solid nutrient agar, at room temperature.

Table I - Bacterial strains.

Strain	Characteristics	Source
<i>Proteus mirabilis</i>		
N17G	plasmidless; isolated from clinical sample	S.O.P. Costa
N17G <i>rif^s nal</i>	<i>rif^s nal</i> mutant from N17G	S.O.P. Costa
L1 to L166	clinical isolates; multiple drug resistance	G.V.A. Pessoa
L167 to L176	freshly isolated clinical samples	G.V.A. Pessoa
L68 N1 to N30	sub-clones from L68	S.O.P. Costa
<i>Escherichia coli</i>		
J53	<i>pro met F</i>	D.S. Santos
HB101	<i>leu pro thi lac gal str recA r⁻ m⁻</i>	D.S. Santos
HB101/68N	HB101 harboring plasmid from <i>P. mirabilis</i> L68 sub-clones	S.O.P. Costa

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Media composition

P. mirabilis and *Escherichia coli* strains were grown in LB, Luria Broth (Miller, 1972) Solid medium was nutrient agar (NA) - Difco Laboratories.

Minimal medium (MM) for *E. coli* was prepared according to Davis and Mingioli (1950) and supplemented with the appropriate nutritional requirements for strain HB101. Minimal medium for *P. mirabilis* had the same basic composition, with nicotinic acid added to a final concentration of 7.5 µg/ml.

Determination of auxotrophy frequency

Determination of auxotrophy in the population was made by replica-plating.

Identification of auxotrophic

Mutants were inoculated on minimal pour plate and the identification of growth requirements was made by "spot tests" with growth factors, according to Davis *et al.* (1980). Alternatively, we also used the technique described by Pontecorvo (1949), in which crystals of each nutritional requirement are added to the surface of MM where the bacterial sample had previously been plated.

Reversion tests

Auxotrophic mutants were grown in 10 ml LB overnight at 37°C, with aeration; the suspension was centrifuged at 7000 rpm for 10 minutes and the bacterial pellet resuspended in 1 ml saline. Aliquots of 100 µl were

plated on MM. The total number of cells was estimated by plating appropriate dilutions on NA plates.

Reversion tests after mutagenic treatment

This test was made essentially as described above, but the mutants were grown in rich medium containing 1.5 mg/ml ethidium bromide for *P. mirabilis* or 0.1 mg/ml ethidium bromide for *E. coli*, according to techniques described by Nakano *et al.* (1980) and Costa *et al.* (1984).

Conjugation experiments

Conjugation was made on solid medium (NA plates); donor and recipient strains were grown to mid-log phase in LB; 0.1 ml of the donor culture was dropped on the center of a NA plate and kept under a hood until complete drying; 0.1 ml of the recipient culture was dropped on top and similarly dried. The resulting plate was incubated at 37°C for 4-18 hours. The conjugation mixture was washed with sterile saline, and aliquots were plated on an appropriate selective medium.

Bacterial transformation

Transformation was made essentially as described by Cohen *et al.* (1973)

RESULTS

Frequency of auxotrophy in *P. mirabilis* strains

We analysed 100 clinical isolated strains of *P. mirabilis*. A minimum of 800 colonies, of each strain, were

Table II - Occurrence of auxotrophy in some *Proteus mirabilis* strains initially found stable for prototrophy and re-evaluated again after four years of storage.

Strain	Colonies analysed	Frequency auxotrophy
L4	985	0.031
L24	1093	0
L74	2681	0.072
L83	1334	0.004
L90	972	0
L96	1108	0.254
L104	1409	0
L126	1013	0.057

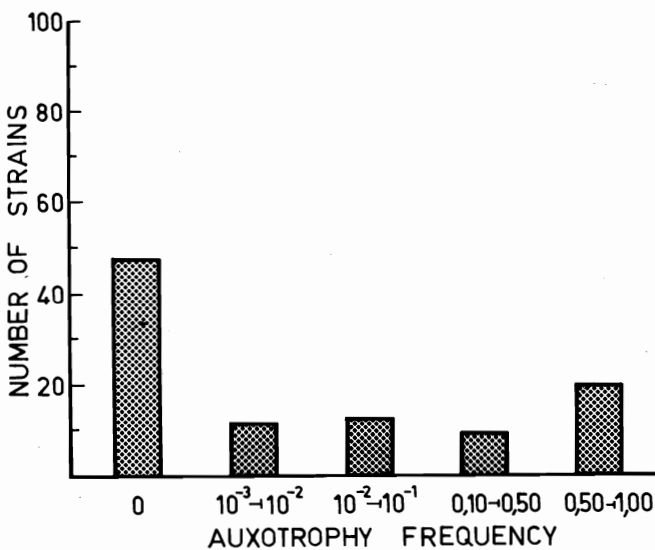


Figure 1 - Frequency of auxotrophy found in 100 strains of *Proteus mirabilis* isolated from clinical material.

studied. Fifty-four percent of these strains had a high frequency of auxotrophy. Prototrophic clones of certain strains gave rise to auxotrophic ones at frequencies as high as 15%. Some of these strains which were found to be stable for prototrophy, were evaluated again after four years, giving the results shown in Table II and Figure 1. All the strains analysed had been kept in our laboratory collection for several years. Table III shows the frequency of auxotrophy found in 10 freshly isolated clinical samples, which have not been kept under storage in the laboratory before testing.

Frequency of auxotrophy from prototrophic clones of highly unstable *P. mirabilis* strains

Strains L43 and L46, kept in our collection showed a high frequency of auxotrophy for carbamoyl phosphate

Table III - Occurrence of auxotrophy in freshly isolated *Proteus mirabilis* strains from clinical materials.

Strain	Colonies analysed	Frequency auxotrophy
L167	1358	0
L168	2163	0
L169	976	5.12×10^{-3}
L170	1231	0
L171	765	0
L172	2081	7.21×10^{-3}
L173	894	0
L174	1512	0.87
L175	968	0
L176	1396	0.33

Table IV - Frequency of auxotrophy from prototrophic sub-clones of strains L43 and L46 of *Proteus mirabilis*

Strain/Sub-clones	Colonies analysed	Frequency auxotrophy
L43. SC1	485	0.109
SC2	805	0.073
SC3	250	0.148
SC4	310	0.155
L46. SC1	794	0.027
SC2	447	0.034
SC3	970	0.029
SC4	832	0.030

synthetase (89 and 98% respectively). Four prototrophic clones of each strain were isolated and analysed for auxotrophy; results are depicted in Table IV.

Identification of auxotrophy of unstable strains

Auxotrophic mutants from unstable strains of *P. mirabilis* were identified by the method of Davis (1980). The majority of these mutants (344 out of 350) were shown to require arginine and uracil for growth in minimal medium. The remaining six mutants were identified as auxotrophic for methionine or cysteine (five mutants) and valine (one mutant). Some auxotrophic mutants for arginine and uracil from different strains, were plated on minimal medium containing: arginine + uracil, ornithine + uracil; citruline + uracil; ornithine only or citruline only, to determine the mutation involved. All mutants analysed grew on plates supplemented either with arginine + uracil or citruline + uracil, suggesting that the mutation involves the gene *car* (carbamoyl phosphate synthetase).

Reversion tests

No reversion to prototrophy could be observed for any of the 10 car mutants analysed. The frequencies of reversion for the two met/cys and one val mutants were about 2.6×10^{-9} . Car mutants which were shown not to revert to prototrophy, were treated with ethidium bromide and submitted to the same reversion test. No reversion to prototrophy could be observed for any of the 10 mutants analysed.

Occurrence of additional auxotrophy in car mutants

Strain L66, found to be 100% auxotrophic for carbamoyl phosphate synthetase, originated additional auxotrophic mutants upon 14 passages in rich medium; out of seven mutants (a total of 4198 colonies analysed), four were identified as dependent on threonine and three as dependent on histidine for growth. All threonine-mutants could be deletions, since no revertants were isolated; histidine mutants reverted at frequencies of 4.2 to 5.4×10^{-9} . The reversion rate was not increased by mutagenic treatment.

Instability of drug-resistance markers in *P. mirabilis*

The clinical isolates used in this work harbor plasmid-encoded resistance to several antibiotics. The markers analysed, ampicillin (Ap), kanamycin (Km), chloramphenicol (Cm), tetracycline (Tc), streptomycin (Sm), trimethoprim (Tp), showed instability; Ap, Km and Cm were lost at high frequencies. We analysed the resistance pattern of subclones from strain L68. Our results indicated that ampicillin resistance is a highly unstable trait, followed by kanamycin and chloramphenicol resistance. Resistance to tetracycline, streptomycin and trimethoprim behaved stably. We had no success in transferring the plasmid (p L68), present in such sub-clones

Table V - Occurrence of auxotrophy in *Escherichia coli* HB 101 harboring the drug-resistance plasmid from *Proteus mirabilis* L68 sub-clones.

Transformant	Resistance pattern	Total analyzed	Total auxotrophic
HB101.68N2	KmTc	2194	0
HB101.68N9	KmTc	2028	0
HB101.68N19	ApKmTcCm	300	8
HB101.68N23	ApKmTcCm	1110	11
HB101.68N26	ApKmTcCm	30	3
HB101.68N27	ApKmTcCm	1235	0

to *P. mirabilis* N17G *rif str nal* or to *E. coli* strain J53 by conjugation. Several sub-clones had their plasmid DNA extracted and used to transform *E. coli* strain HB101. The transformants, selected with ampicillin or tetracycline, had their resistance pattern analysed, and were checked for occurrence of auxotrophy. Out of 22 mutants, 14 were dependent on tyrosine and eight on arginine and uracil for growth (Table V). Reversion tests made on car and tyrosine mutants from *E. coli* HB101 showed that both mutations revert to prototrophy (2.9 to 5.1×10^{-4} and 1.5 to 1.6×10^{-5} respectively).

Occurrence of additional auxotrophic mutants in *E. coli* HB101 carbamoyl phosphate synthetase and tyrosine requiring mutants

The mutants isolated had their populations analysed for the presence of additional auxotrophic mutants. A total of 9738 colonies from carbamoyl phosphate synthetase requiring strains and 18039 colonies from tyrosine requiring strains were analysed. No additional auxotrophic mutants were found.

Plasmid-curing from HB101 derivatives

Strain HB101/pL68 car^+ and car^- was treated with acridine orange (50 mg/ml). Elimination of all resistance markers occurred in ca. 95% of car^+ clones and ca. 98% of car^- clones.

DISCUSSION

We found a novel phenomenon of high genetic instability related to auxotrophy in strains of *P. mirabilis*. Analysis of 100 clinical samples of *P. mirabilis* kept in our laboratory collection showed that 54% of the samples present auxotrophy at frequencies higher than 10^{-3} . Four years after the initial analysis, eight samples which were 100% prototrophic were re-evaluated, and five of the samples were shown to present auxotrophy at frequencies of 0.004 to 0.254. Since these samples had been kept in our laboratory for several years, we analysed 10 freshly isolated *P. mirabilis* strains. This analysis showed four samples with a high, frequency of auxotrophy, suggesting that this phenomenon is not linked to long term storage, and occurs widely in this species.

The level of genetic instability is further demonstrated by the analysis of populations originated from prototrophic clones isolated from strains populations with a high frequency of auxotrophy. This high frequency of auxotrophy cannot be explained by the usual rates of mutation (generally around 10^{-7} ; Kimura and Ohta, 1973).

The identification of 350 mutants revealed an interesting feature: over 98% of them are dependent on

arginine and uracil for growing on minimal medium. They are also able to grow on minimal medium supplemented with citruline plus uracil, suggesting that the mutation affects the gene *car* (carbamoyl phosphate synthetase EN.2.7.2.29). In *E. coli*, there are two genes involved in the synthesis of this enzyme, *carA* and *carB*. The structure of the *P. mirabilis* genome as concerns such genes is not known. The remaining mutants were dependent on methionine/cysteine or valine. Therefore, the high genetic instability seen in *P. mirabilis* strains seems to have a hot spot in the genome, since the absolute majority of the mutants isolated have the same auxotrophic marker.

The *car* mutation behaves as a deletion, since no revertants were found for any of the mutants analysed our experiments would detect reversion rates as low as 10^{-12} . On the other hand, the mutants identified as *met/cys* or *val* reverted at frequencies around 10^{-9} . This frequency was not enhanced by treatment with ethidium bromide. The high frequency of mutation affecting particular points of the genome, with the reversion rate not increased by mutagenic agents, suggests the involvement of transposable elements as agents of instability (Iyobe *et al.*, 1970; Saedler *et al.*, 1978; Hom *et al.*, 1984).

The clinical isolates used in this work have multiple drug-resistance markers which are plasmid-encoded. These markers are lost at high frequencies, a phenomenon probably associated with plasmid rearrangements quite common in *P. mirabilis* (Coetzee, 1972).

Our attempts to transfer by conjugation the plasmid present in sub-clones of L68 were not successful. We tried to do so using the plasmidless *P. mirabilis* strain N17G as a recipient, as well as *E. coli* strain J53. However, the plasmid was easily introduced by transformation into *E. coli* strain HB101. This strain is chromosomally resistant to streptomycin, therefore this marker could not be analysed in our experiments. *P. mirabilis* harboring only TcSmTp as resistance markers do not originate transformants. It is possible that these markers are not plasmid-encoded in such sub-clones. We have never been able to visualize the plasmid DNA from *P. mirabilis* in agarose gels, although several different protocols have been used. Visualization is possible after introduction in *E. coli* HB101.

Transformants of HB101 with plasmids from L68-derivatives were analysed for auxotrophy. We found instability at frequencies ranging from 0.009 to 0.100. The mutants isolated were identified as tyrosine-dependent and arginine plus uracil-dependent. Both types of mutants reverted at frequencies not increased by mutagenic treatment. We have not been able to detect spontaneous auxotrophic mutants in HB101 not harboring the *P. mirabilis* plasmid. Therefore, we have been able to induce a similar genetic instability in *E. coli*, upon transfer of a

drug-resistance plasmid from *P. mirabilis*. The generated instability had a different behavior concerning the genome target, since two genes had been similarly affected, *tyr* and *car*. Furthermore, neither mutation behaved as a deletion. Different to what had been found for *P. mirabilis*, no additional mutations could be detected in tyrosine and carbamoyl phosphate synthetase auxotrophic clones from HB101.

We next attempted to eliminate the plasmid from *car*⁺ and *car*⁻ HB101 clones, to verify if the mutation could be caused by transposon-insertion. However, the plasmid was cured from the strains, with no retention of any drug-resistance marker. This result ruled out the involvement of a transposon in the auxotrophic mutation. The possible involvement of an IS in such instability is under investigation.

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RESUMO

Relata-se um novo fenômeno de instabilidade genética em amostras de *Proteus mirabilis* relacionado com auxotrofia. *P. mirabilis* contendo plasmídeo R, estocados no laboratório ou recentemente isolados de material clínico mostraram que 54% deles apresentam auxotrofia em frequência maiores do que 10^{-3} . Colônias prototróficas de algumas amostras dão origem a colônias auxotróficas em frequências próximas a 15%. Essa instabilidade afeta principalmente o gene *car* da carbamoyl fosfato sintetase, que leva à dupla deficiência nutricional - arginina e uracila. Outros genes são também afetados mas em baixa frequência. A mutação *car* não reverte à prototrofia. Um fenômeno similar de instabilidade pode ocorrer em *Escherichia coli* HB101 após a introdução do plasmídeo R de *P. mirabilis*. Foi afastada a hipótese de um transposon causar essa auxotrofia.

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