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Biochemical characterization and genetic mapping of purine genes in *Micrococcus luteus*

By NIRUPAMA MOHAPATRA and WESLEY E. KLOOS
Department of Genetics, North Carolina State University, Raleigh, North Carolina 27607

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SUMMARY

Purine auxotrophs of *Micrococcus luteus* were induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. They were biochemically characterized by growth stimulation with CO_2-enriched air and purine intermediates and by the Bratton-Marshall test that is used to detect accumulated diazotizable amines. The mutants were divided into four classes: *pur*(*EC*), *purH*, *purJ*, and *Pur*$^-$. The *pur*(*EC*) class was further subdivided into *purE* and *purC* by results of crossing all the *pur*(*EC*) mutants with a known *purE* reference strain (ATCC 27141). Mutants which were not linked to the reference *purE* marker were considered to be *purC*. Genetic mapping was accomplished by using two-point reciprocal transformation crosses. The result of this study indicates that *purJ* mutants may be loosely linked to *purE* and *purC*, and purH mutants are not linked to *purE*, *purC* or *purJ*.

1. INTRODUCTION

Purine biosynthesis has been studied extensively in avian and mammalian systems (Buchanan & Hartman, 1959; Schulman, 1961). Among micro-organisms, many studies of purine biosynthesis have been conducted in *Escherichia coli* (Gots & Gollub, 1957; Gollub & Gots, 1959; Tritz, Matney, Chandler & Gholson, 1970), *Salmonella typhimurium* (Yura, 1956; Gots, Dalal & Shumas, 1969; Gots, Benson & Shumas, 1972) and *Aerobacter aerogenes* (Magasanik & Brooke, 1954; Balis *et al.* 1956; Magasanik & Karibian, 1960). Baxter-Gabbard & Pattee (1970) biochemically characterized eight different classes of purine auxotrophs in *Staphylococcus aureus*.

In *S. typhimurium* ten different genes controlling purine biosynthesis were separated into one cluster of three genes, one cluster of two genes and five unlinked single genes (Demerec, 1964). Similar results were obtained for *E. coli K12* where 11 genes were mapped by conjugation (Stouthamer, De Haan & Nijkamp, 1965). Taylor & Trotter (1967) and Sanderson (1967) revised the linkage maps of *E. coli* and *S. typhimurium*, respectively, and reported that in both organisms most of the purine genes are scattered over a large region of the linkage map. Genetic and

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biochemical studies of *Micrococcus luteus* were undertaken to compare the chromosomal arrangement of purine genes with those of other organisms and to compare steps in purine biosynthesis.

2. MATERIALS AND METHODS

(i) *Bacterial strains*

A purine-independent isolate (*purE*$^+$) of *M. luteus* strain *ATCC 27141* was the source of all purine auxotrophs. Reference purine mutants of *S. typhimurium*, denoted as *purE11*, *purB12* and *purH340*, were obtained through the courtesy of Dr J. S. Gots, University of Pennsylvania, Philadelphia, Pa.

(ii) *Mutagenesis*

An 18 h slant of strain *ATCC 27141* (*purE*$^+$) was suspended in 5 ml sterile saline and centrifuged. The harvested cells were resuspended in *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) solution and incubated for 60 min at 34 °C. The NTG solution was prepared in Na-citrate buffer, pH 5·0, at a concentration of 300 μg/ml and filter-sterilized. The treated cells were centrifuged, washed, and resuspended in saline. A series of dilutions were made and plated to test cell viability. Appropriate cell dilutions of the saline suspension were then used to isolate the mutants.

(iii) *Media and culture conditions*

M. luteus and *S. typhimurium* strains were maintained on P-agar (Naylor & Burgi, 1956) slants at 4 °C. *M. luteus* defined broth and agar media were prepared as described by Kloos (1969*a*). Purine mutants of *S. typhimurium*, used as standards in accumulation tests, were grown in the glucose-salt medium E of Vogel and Bonner (1956) at 37 °C. *M. luteus* was cultured at 34 °C in all experiments.

(iv) *Biochemical characterization*

Suspected purine auxotrophs were tested for their growth response to different purine intermediates and derivatives. All the auxotrophs were plated on defined agar medium and a few crystals of adenine, guanine, adenosine, xanthosine, xanthine, hypoxanthine, inosine, 5-amino-4-imidazolecarboxamide (AICA) and AICA-riboside were added separately. Generally, two chemicals were used per plate maintaining safe distance between the chemicals to avoid their interaction.

Many intermediates in the purine pathway are derivatives of amino-imidazoles and the amino groups of these compounds can be diazotized. Bratton and Marshall's colorimetric procedure described by Flaks & Lukens (1963) was used to test the presence of the aminoimidazoles such as 5-aminoimidazole ribonucleotide (AIR), 5-amino-4-imidazole-*N*-succinocarboxamide ribonucleotide (succino-AICAR), and 5-amino-4-imidazolecarboxamide ribonucleotide (AICAR) in the culture fluids of the purine auxotrophs.

Test for growth stimulation under CO_2-enriched air was carried out as described by Charles (1962). All the purine auxotrophs were inoculated on unsupplemented

defined agar plates and incubated under 25 % CO_2 + 75 % air at 34 °C for 72 h. Another set of plates was incubated under air.

(v) *Transformation crosses*

The genetic symbols used to designate the *M. luteus* auxotrophs are those used by Tritz *et al.* (1970) for *E. coli*. Two-point transformation crosses were performed according to the procedure of Kloos & Rose (1970). DNA from donor strains for transformation was isolated as reported by Kloos (1969*b*), with the exception that the ribonuclease treatment was omitted. The unlinked reference marker strain used in this study was a tryptophan auxotroph of *M. luteus*, denoted *trpC23*.

Table 1. *Biochemical characterization of purine auxotrophs of* Micrococcus luteus

Mutant class	Representative strains	Growth response	Terminal accumulated intermediate
pur(*EC*)	4, 5, 8, 11, 12, 13, 14, 15, 17, 35, 39, 51, 54, 55, 59, 61, 62, 72, 73, 74, 82, 84	Adenine, adenosine, hypoxanthine, inosine, 5-amino-4-imadazole carboxamide (AICA), no growth on guanine	5-Amino imidazole ribonucleotide (AIR)
purH	1, 40, 43, 46, 47, 64, 83	Adenine, adenosine, hypoxanthine, inosine	5-Amino-4-imadazole carboxamide ribonucleotide (AICAR)
	28, 44, 50, 52, 58, 86, 88	Hypoxanthine, guanine, inosine, adenine, adenosine	
purJ	7, 19, 30, 60, 69, 81	Adenine, adenosine, hypoxanthine, guanine, inosine	5-Formamido-4-imidazole carboxamide ribonucleotide (FAICAR)
Pur⁻*	10, 33, 53, 57, 66, 80, 85, 87	Adenine, adenosine hypoxanthine, inosine	None
	22, 45, 70, 79	Adenine, adenosine, hypoxanthine, inosine, guanine	None

* Pur⁻ mutants 57, 22, 70 and 79 also were stimulated by AICA.

Each transformation cross was repeated three times and the number of plates prepared for each cross varied from three to five depending upon the competency of the recipient. The final data are the average of three replicates. Determination of linkage and estimation of relative distance between the recipient and the donor markers was based on the calculation of a two-point recombination index according to the following equation (modified from Chapman and Nester (1969) to include single mutants):

$$\frac{\text{prototrophs from } purX \times purY/\text{prototrophs from } trpC23 \times purY}{\text{prototrophs from } purX \times \text{WT}/\text{prototrophs from } trpC23 \times \text{WT}}.$$

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3. RESULTS

(i) *Biochemical characterization*

Eighty-eight purine mutants were initially isolated in this study. The transformation competency of the induced purine mutants was usually low, and several mutants failed to demonstrate detectable competency. Some strains also demonstrated a high reversion to prototrophy. Of the original auxotrophs isolated, 54 were chosen for further investigation.

Growth of all purine mutants was stimulated by adenosine, adenine, hypoxanthine, or inosine (Table 1), thus indicating that the block in these mutants was

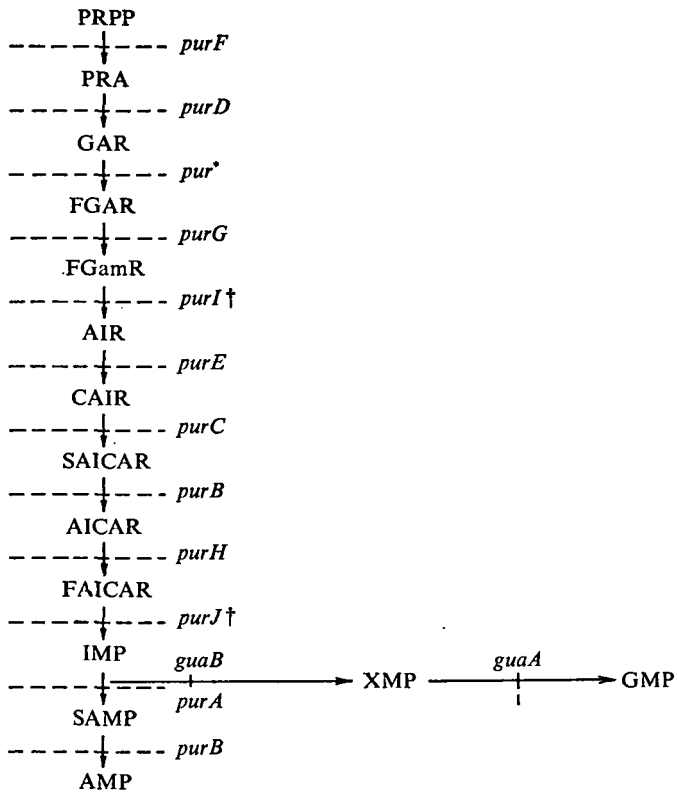

Fig. 1. Purine biosynthetic pathway in enteric bacteria (according to Tritz *et al.* 1970). Abbreviations: PRPP, 5-phosphoribosyl-pyrophosphate; PRA, 5-phosphoribosylamine, GAR, glycinamide ribonucleotide; FGAR, formyl glycinamide ribonucleotide; FGamR, formyl glycinamidine ribonucleotide; AIR, 5-aminoimidazole ribonucleotide; CAIR, 5-amino-4-imidazolecarboxylic acid ribonucleotide; AICAR, 5-amino-4-imidazolecarboxamide ribonucleotide; SAICAR, 5-amino-4-imidazole-*N*-succinocarboxamide ribonucleotide; FAICAR, 5-formamido-4-imidazolecarboxamide ribonucleotide; IMP, inosinic acid; AMP, adenylic acid; SAMP, adenylsuccinic acid; XMP, xanthylic acid; GMP, guanylic acid. Genes coding enzymes at each step in the pathway are designated according to the scheme in *Escherichia coli* (Taylor & Trotter, 1967). *This gene has not been mapped in either *S. typhimurium* or *E. coli*. †These genes have been mapped in *Salmonella typhimurium* but not in *E. coli*.

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before the formation of inosine-5-phosphate (IMP) (Fig. 1, Table 1). About one-third of the mutants tested were stimulated by guanine, xanthine or xanthosine. The other two-thirds of the mutants, although blocked before the formation of IMP, did not grow on guanine, xanthine or xanthosine. These mutants may be unable to take up guanine and/or could not convert guanine to another purine due to a lack of phosphoribosyl transferase activity. Gots *et al.* (1972) have reported this class of mutation in *S. typhimurium proAB purE* which they have designated as *gxu*. When AICA was added to the growth medium, it stimulated slow but distinct growth for some mutants, thus indicating that these mutants were blocked before the formation of Succino-AICAR (Fig. 1).

Table 2. *Biochemical characterization of* pur(EC) *mutants*

Representative strain	Terminal accumulation	CO_2 stimulation	Mutant designation
8, 15, 35, 51, 61, 62, 73, 82	5-Amino imadazole ribonucleotide (AIR)	+	*purE*
4, 5, 11, 12, 13, 14, 17, 39, 54, 55, 59, 72, 74, 84	AIR	−	*pur(EC)*

The culture fluid of each purine auxotroph was tested for the presence of aminoimidazole by the Bratton-Marshall test. The culture fluid of mutants which developed an orange color in the Bratton-Marshall test and gave an absorption maximum at 500 nm, characteristic of AIR, were considered to be either *purE* or *purC* mutants (Stouthamer *et al.* 1965). Because both classes of mutants accumulate the same compound, they cannot be distinguished and are, therefore, designated *pur(EC)*. Mutants whose culture fluid produced a purple colour in the Bratton-Marshall test with an absorption maximum at 540 nm, characteristic of AICAR, were designated *purH*. Mutants which did not accumulate Bratton-Marshall positive compounds and mutants which showed only a slight accumulation of AICAR were tested for 5-formamido-4-imidazole-carboxamide ribonucleotide (F-AICAR). This was accomplished by acid hydrolysis of the culture fluid followed by the Bratton-Marshall test for AICAR. Mutants which were positive for this test were designated *purJ* (Gots, Dalal & Shumas, 1969) and those which were not positive or further defined were designated as Pur$^-$ mutants (Table 1).

Since CO_2-enriched air can stimulate growth of some leaky purine mutants of *Neurospora crassa* blocked in the conversion of AIR to 5-amino-4-imidazole-carboxylic acid ribonucleotide (CAIR) on minimal media (De Serres, 1966), we attempted to see if mutants of *M. luteus* could be resolved using this test (Table 2). To accomplish this we tested all purine mutants, but were particularly interested in resolving those designated as *pur(EC)*. While lack of stimulation under CO_2-enriched air does not mean that the mutants are not *purE*, the reverse is a good confirmation.

(ii) *Separation of* purE *and* purC *mutants by genetic analysis*

Because all *purE* and *purC* mutants could not be separated by intermediate stimulation or accumulation, genetic analysis was used to identify these mutants. Since *purE* and *purC* loci are unlinked in *E. coli* (Taylor & Trotter, 1967) and *S. typhimurium* (Sanderson, 1967), a similar situation might exist in *M. luteus*. If this were the case, both mutant groups could be separated by crosses with

Table 3. *Separation of* pur(EC) *mutants into* purE *and* purC *by crossing to a reference* purE *mutant ATCC* 27141

Donor DNA *pur(EC)*	Recombination index	Redesignated mutant class
5, 8, 14, 15, 35, 39, 51, 54, 55, 61, 62, 73, 82, 84	0·01–0·22	*purE*
4, 11, 12, 13, 17, 53, 59, 72, 74	0·86–1·93	*purC*

reference *purE* or *purC* mutants. Accordingly, one known *purE* mutant (ATCC 27141) of *M. luteus* was used as the recipient and all the *pur(EC)* mutants were used as donors in two-point transformation crosses. This resulted in two different patterns of recombination indices. Mutants which were very closely linked to the reference *purE* marker gave very low recombination indices (0·01–0·22) and were classified *purE*. Mutants with high recombination indices (0·86–1·93) were considered not to be linked to *purE* and were therefore classified *purC* (Table 3). These genetic data are confirmed by CO_2 stimulation results since all mutants showing growth stimulation under CO_2-enriched air were very closely linked.

(iii) *Linkage relationships of purine genes*

Pur$^-$ and *purH* mutants were not used as recipients in crosses because of their lack of competency. To determine the linkage relationships of the remaining purine mutants, two mutants from each biochemical class were used as recipients with all mutants as donors. The average of all recombination indices from each specific set of crosses was compiled and a reciprocal cross table was constructed (Table 4). Markers that produced low recombination indices (0·02–0·2) belonged to the same biochemical class. Markers showing recombination indices between 0·3–0·8 might be loosely linked to each other and were in different biochemical classes. These markers, however, failed to provide evidence for cotransformation. Recombination indices above 0·8–1·0 suggest that the markers are unlinked.

(iv) *Tentative mapping of loosely linked purine genes by two-point reciprocal crosses*

Mapping of different purine genes that appear to be loosely linked was done by the reciprocal crossing of two mutants from each group of mutants, i.e. *purE*, *purC* and *purJ* (Table 5). Based on the data presented in Table 4, arrangement of

purine genes in *M. luteus* and the relative distance between them was derived (Fig. 2). Although Table 4 lists the recombination indices of Pur⁻ donors with *purE*, *purJ* and *purC* recipients, Pur⁻ is not placed in Fig. 2 because the linkage relationship among the Pur⁻ mutants is not characterized in relation to each other due to their lack of competency.

Table 4. *Average recombination indices of crosses between various purine mutants*

	Donor				
Recipient	*purE*	*purJ*	*purC*	*purH*	Pur⁻
purE	0·08	0·78	1·23	1·94	1·40
purJ	0·58	0·10	0·47	1·75	2·38
purC	1·24	0·56	0·13	1·20	1·94

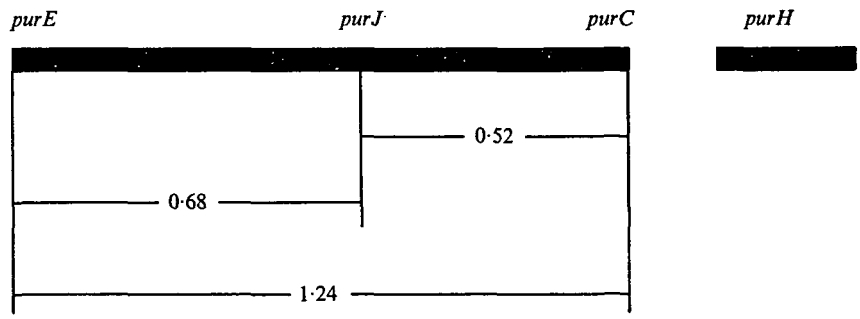

Fig. 2. Purine gene arrangements in *M. luteus* as determined by the average recombination indices of individual mutant crosses. *PurH* is not linked with *purE*, *purJ* or *purC*. Data for crosses are shown in Tables 4 and 5.

4. DISCUSSION

Four different purine genes, each presumably representing a step (enzyme) in purine biosynthesis, were mapped. One group of mutants which could not be identified either by auxanography or detectable accumulations was designated as Pur⁻. These Pur⁻ mutants are thought to be blocked before the formation of AIR.

Since two-point crossing was the only expedient method available for mapping purine loci, the concept of 'best-fit' method of Hartman, Loper & Serman (1960) was used in constructing a tentative map order. As shown in Fig. 2, the purine genes are not clustered on the chromosome of *M. luteus*. There is a possible loose linkage between *purE*, *purJ* and *purC*. The general wide chromosomal separation of purine genes of *M. luteus* is rather similar to the situation found with *E. coli* and *S. typhimurium*, though the possible loose linkage relationship of *purJ* with *purE* and *purC* is different. Genetic analysis of *purH* and *purJ* mutants of *S. typhimurium* reported by Gots *et al.* (1969) revealed close linkage between these two loci. The data obtained from the genetic analysis of *purH* and *purJ* mutants of *M. luteus* indicate that these loci are not linked (Table 4).

Table 5. *Recombination indices of reciprocal crosses between* purE, purJ *and* purC *mutants*

Recipient	Donor					
	ATCC 27141 (*purE*)	*E15*	*J60*	*J81*	*C4*	*C59*
ATCC 27141 (*purE*)	0	0·03	0·77	0·73	1·73	1·57
E15	0·01	0	0·74	0·57	1·11	0·90
J60	0·47	0·68	0	0·17	0·37	0·37
J81	0·66	0·47	0·24	0	0·62	0·51
C4	1·55	1·27	0·45	0·57	0	0·06
C59	0·97	1·35	0·58	0·64	0·05	0

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