ISOLATION OF CYSTEINE AND METHIONINE AUXOTROPHIC MUTANTS IN *SINORHIZOBIUM MELILOTI*

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ABSTRACT

Random Tn5 mutagenesis produced 21 cysteine/methionine and 13 methionine auxotrophic mutants of *Sinorhizobium meliloti* strain Rmd201. Linkage of Tn5 to auxotroph indicated that each mutant had a single Tn5 insertion. All auxotroph showed spontaneous reversion to prototrophy and they resembled the parental strain in production of cell surface molecule and utilization of sugars and dicarboxylic acid.

INTRODUCTION

Nitrogen fixing bacteria like *Klebsiella pneumoniae* and *Azotobacter* are able to reduce atmospheric nitrogen into ammonia as free living organisms. Other organisms fix nitrogen only in symbiotic relationship with an eukaryotic host plant, like rhizobia in legume symbiosis (1). The detail knowledge of the gene involved in symbiosis will be of great help in obtaining symbiotic combinations having enhanced ability of nitrogen fixation.

Rhizobial cell surface molecules which play an important role in infection of the legume host plant, is composed of exopolysaccharides (EPS), lipopolysaccharides (LPS), β-glucans and capsular polysaccharides (KPS). Normal expression of the genes for cell surface molecules is required for rhizobium-legume symbiosis (2,3,4,5,6).

Sulfur-containing amino acids auxotrophs of rhizobia have been isolated by several workers (7,8,9). Methionine and cysteine/methionine mutants of *S. meliloti* strain 2011 were found to induce effective nodules on alfalfa plant (10). Cystein-requiring mutants of *S. meliloti* strain L5-30 showed loss of effectivness (11). Kerppola and Kahn (12) observed that the methionine mutants of *S. meliloti* strain 104A14 formed ineffective nodule on alfalfa. *Rhizobium etli metZ* mutant was unable to produce lipochito-oligosaccharides or induce nodules on root of *Phaseolus vulgaris* (13).

MATERIAL AND METHODS

Strains and plasmids: The bacterial strains and plasmid used in this study are listed in table 1.

Media and supplemerts: Complete medium trypton yeast extract (TY) and rhizobial minimal medium (RMM) have been described ealier (14). Stock solutions were added to the autoclaved media to make final concentrations of 50, 30 and 10 mg/ml from amino acids, nitrogen bases and vitamins, respectively.

Antibiotics used were purchased from HiMedia Laboratories. Streptomycine sulfate and kanamycine acid sulfate stock solutions were prepared in sterile distilled water. The final concentration of antibiotics used in different media were as follows: streptomycine sulfate (100 μg/ml; kanamycine acid sulfate (100 μg/ml for *E. coli* and

400 µg/ml for *S. meliloti*), Clorompinicol (50 µg/ml), rifampicin (40 µg/ml) and tetracycline hydrochloride (15 µg/ml).

Transposon Tn5 mutagenisis: Bacterial conjugations were don according to Kondorosi *et al* (15). Random mutagenesis of *S. meliloti* strain Rmd 201 was carried out using Tn5 delivery suicide plasmid pGS9 (16). Transconjugants obtained were screened for auxotrophs by streaking them on RMM and TY+Km^{400}+Sm^{100} agar medium. The auxotrophy of each strain was determined on the modified Holliday pools (17).

Linkage of Tn5 insertion to auxotrophy: Transposon Tn5 encoded kanamycin resistance marker from each auxotroph was mobilized to *S. meliloti* ZB557 strain with the help of genome mobilizing plasmid pJB3JI.

Reversion analysis of auxotrophs: Spontaneous reversion frequency for the auxotroph was calculated on the basis of the number of prototrophic revertants (number of colonies on RMM) and the total number of cell (number of colonies on TY medium), after streaking the same volume on each medium.

Test for production of cell surface molecules: The test for production of lipopolysaccharides, the test for production of succinylated exopolysaccharides, the test for production of celluose fibrils, the test for production of β -(1-3)-glucans nad the test for production of β -(1—2)-glucans were don as describe earlier (18).

RESULTS AND DISSCUSION

Isolation of auxotrophs: Three thausand transposon Tn5 derivatives (Km^r) of *S. meliloti* strain Rmd201 were obtained and out of these 12 did not grow on *Rhizobium* minimal medium (RMM). Three sulfur amino acid auxotrophs isolated during this study and two such auxotrophs generated previously by other workers were used for further studies. The auxotrophic mutants with their requirements were listed in table 2. Isolation of auxotrophs which grow on cystein or methionine has also been reported by several workers (7,13). Methionine auxotroph cannot grow on RMM supplemented with cysteine. It seems that *S. meliloti* cannot convert cysteine to methionine.

Linkage of Tn5 insertion to auxotrophy: All kanamycin resistant transconjugants showed the respective donors auxotrophic marker, hence, there was 100% cotransfer of Tn5 and the auxotrophic marker. This indicate that the Tn5 insertion is responsible for auxotrophy in each strain.

Reversion analysis: All auxotrophic mutants showed spontanous reversion to prototrophy with a percentage range between $4.5x10^{-8}$ to $4.5x10^{-6}$ (table 3). The revertants did not grow on TY medium containing kanamycin. This indicates the excision of transposon Tn5 from the genome.

Production of cell surface molecules:

The results of production of cell surface molecule are listed in table 4. All auxotrophic mutants, like the parental strain Rmd201, produced lipopolysaccharides (LPS), succinylated exopolysaccharides (EPSI), cellulose fibrils, β -(1-2)-glucan and β -(1-3)-glucan. It seems that the genes involved in cysteine and methionine synthesis is not responsibe for cell surface molecule production.

Utilization of sugars and dicarboxylic acids:

No change in the growth behavior of any auxotroph was detected when glucose in RMM was replaced by any of the other sugars (arabinose, maltose, sorbitol, manitol, or sucrose) or dicarboxylic acids (malic acid, asparticacid or sodium succinate) as carbon source. The presence of functional C_4-dicarboxylic acid transport system of *R. leguminosarum* was found to be essential for nitrogen fixation (19).

Table 1: Bacterial strains and plasmids used

Strain/plasmid	Relevant characteristics	source/reference
Sinorhizobium meliloti		
AK631	Nod+Fix+, compact colony varient of wild type Rm41	Adam Kondorosi
Rmd201	spontaneous Sm^r derivatives of AK631	Khanuja &kumar (20)
PP631	AK631(pJB3J1)	Peter Putnoky
ZB557	Rm41 *Phe15 leu4 rfl sm1*	Peter Putnoky
BA4, BA7	Rmd201 cysI/cysJ::Tn5	This study
BA8	Rmd201 metA/metZ::Tn5	This study
VK29	Rmd201 metE::Tn5	Vineetha KE & Prasad CK
VK39	Rmd201 metF::Tn5	Vineetha KE & Prasad CK
Escherichia coli		
WA803 (pGS9)	Met- Thi- Cmr Kmr	Selvaraj & Iyer (16)
Plasmids		
PGS9	IncN repP15A Cmr Kmr	Selvaraj & Iyer (16)
PJB3J1	Kms derivative of pR68.45, capable of mobilizing genomic segments of its host, Tcr Cbr Nalr	Brewin *et al.*(21)

Table 2. Auxotrophic mutants of *Sinorhizobium meliloti* strain Rmd21 and their nutritional reqiurements.

Strain	Nutritional requirement
BA4	Cysteine or methionine
BA7	Cysteine or methionine
BA8	Methionine
VK29	Methionine
VK39	Methionine

Table 3. Reversion frequencies of auxotrophic mutants of *Sinorhizobium meliloti* Rmd201.

S. meliloti strain	Reversion percentage
BA4	$4.5x10^{-8}$
BA7	$3.7x10^{-8}$
BA8	$3.2x10^{-8}$
VK29	$3.7x10^{-8}$
VK39	$4.5x10^{-6}$

Table 4. Production of cell surface molecules by *Sinorhizobium meliloti* parental strain Rmd201 and its auxotrophic mutants.

Strain	LPS	EPSI	Cellulose Fibrils	β -1-2-gluan	β -1-3-glucan
Rmd201	+	+	+	+	+
BA4	+	+	+	+	+
BA7	+	+	+	+	+
BA8	+	+	+	+	+
VK29	+	+	+	+	+
VK39	+	+	+	+	+

عزل طافرات العوز الغذائي الامينيين السستين والمثيونين في بكتريا السينورايزوبيوم ملبلوتي

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الخلاصة

باستخدام الترانسبوسون Tn5 تم الحصول على ٢١ طافر للحامض الاميني سيستين او ميثيونين و ١٣ طافر للحامض الاميني ميثيونين في بكتريا سينورايزوبيوم ملبلوتي السلالة Rmd201 . لقد اكد ارتباط العوز الغذائي للطافر مع Tn5 ادخال Tn5 واحد الى DNA البكتريا . بينت الدراسة ان كل الطافرات كان لها قابلية الارتداد الى الوضع الطبيعي وكانت الطفرات متشابهة للسلالة الابوية في انتاج بعض المواد السطح خلوية وكذلك في استغلال بعض السكريات والاحماض ثنائية الكربوكسيل .

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