Genetic study of biotin biosynthesis in the obligate methanol-oxidizing bacterium '*Methylobacillus flagellatum*'

I. G. Serebriiski and Y. D. Tsygankov

Author for correspondence: I. G. Serebriiski. e-mail: lab1@vnigen.msk.su

Laboratory of Genetics of Methylotrophic Micro-organisms, Institute of Genetics and Selection of Industrial Micro-organisms, 1st Dorozhny proezd 1, Moscow 113545, Russia

Twenty-seven mutants defective in the biosynthesis of biotin were generated by *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine treatment of the obligate methylotrophic bacterium '*Methylobacillus flagellatum*'. The metabolic lesion in some of these mutants was determined in cross-feeding experiments using known *Escherichia coli* *bio* mutants. R-prime plasmids carrying various fragments of the '*M. flagellatum*' chromosome were used for complementation of *bio* mutations in '*M. flagellatum*' and *E. coli*. By complementation analysis and cross-feeding experiments, '*M. flagellatum*' Bio⁻ mutants were classified into five groups: *bioA*, *bioB*, *bioD*, *bioH*, and a group with unidentified *bio* mutations (*bioF* or/and *bioC*). Using R-prime complementation mapping and also Hfr-like mapping, most of the *bio* genes of '*M. flagellatum*' identified in this study were localized on the '*M. flagellatum*' chromosome.

Keywords: '*Methylobacillus flagellatum*', biotin biosynthesis, obligate methylotroph, *in vivo* cloning, mutant selection

INTRODUCTION

Recently, the genetics of biotin biosynthesis has been extensively studied in enterobacteria and bacilli. It has been shown that six structural genes are specifically involved in the biotin synthetic pathway in *Escherichia coli* (Barker & Campbell, 1980). Products of the genes *bioA*, *bioB*, *bioD* and *bioF* carry out the bioconversion of pimelyl-CoA into biotin (Eisenberg, 1987). Two other genes, *bioC* and *bioH*, are involved in pimelyl-CoA synthesis (Fig. 1). In bacilli, the last four steps are analogous to those in *E. coli* (Pai, 1975). In addition, three new genes have been identified: *bioW*, which encodes pimelyl-CoA synthetase, and *bioX* and *bioY*, encoding proteins with unknown functions (Gloeckler *et al.*, 1990). The classical operon model of Jacob and Monod is applicable to the biotin biosynthetic (*bio*) operon, which consists of the genes *bioABFCD*, in enterobacteria (Eisenberg, 1987; Sakurai *et al.*, 1993). The *bio* locus in *E. coli* maps near the lambda attachment site at 18 min on the chromosome, while the unlinked gene, *bioH*, is located at 75 min (Rolfe & Eisenberg, 1968).

At present, little is known about the biosynthesis of biotin in methylotrophs. Genetic analysis of these bacteria by traditional means has only recently appeared possible, as mutants were difficult to obtain and chromosome-mobilizing plasmids were rare (Holloway, 1984). An approach

called complementation mapping has been used for isolation and characterization of extended genome fragments of *Methylophilus methylotrophus* and '*Methylophilus viscogenes*' (Moore *et al.*, 1983; Lyon *et al.*, 1988).

'*Methylobacillus flagellatum*' is a Gram-negative obligate methylotroph able to use methanol and methylamine as sole sources of carbon and energy. Considerable progress has been made in the physiological, biochemical and genetical characterization of this bacterium (Gomelsky *et al.*, 1990; Tsygankov *et al.*, 1990a, b; Kiriukhin *et al.*, 1993). Here we describe the isolation of Bio⁻ mutants of '*M. flagellatum*', their characterization, and mapping of some *bio* mutants by classical and 'surrogate' (Holloway, 1984) genetics.

METHODS

Bacterial strains and plasmids, growth and mutagenesis conditions. The *E. coli* and '*M. flagellatum*' strains employed are listed in Table 1. Methylotrophic bacteria were cultured in a previously described minimal medium (Tsygankov & Kazakova, 1987) with methanol (M9M) as sole source of carbon and energy. *E. coli* was grown in LB medium in the presence of appropriate antibiotics. Optimal conditions described earlier (Tsygankov & Kazakova, 1987) were used for *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (NTG) mutagenesis of '*M. flagellatum*'.

Cross-feeding experiments. Testing agar plates were prepared

Fig. 1. Biotin biosynthetic pathway in *E. coli* and *Bacillus sphaericus*.

with M9 medium, containing glucose (1%, w/v), agar (0.6%), vitamin-free Casamino acids (2 g l⁻¹) and 2,3,5-triphenyl tetrazolium blue (50 mg l⁻¹), and washed cells of the corresponding Bio⁻ mutant of *E. coli*. After the medium had solidified, drops of supernatants of tested cultures, grown overnight in M9 without biotin, were placed onto the plate. When biotin or its biosynthetic intermediates were excreted by '*M. flagellatum*' in quantities large enough to cross-feed a test strain, its growth was observed by the appearance of a blue colour, resulting from reduction of tetrazolium dye. The size of the zone was dependent on the concentration of biotin (or its biosynthetic intermediate) and the length of the incubation period.

Conjugational crosses. These were carried out as described earlier (Tsygankov *et al.*, 1990a). Derivatives of *E. coli* mutant strains, containing a *recA* mutation, were obtained in conjugational crosses using *E. coli* HfrKL16 *recA56 stlC::Tn10* as a donor. R-prime plasmids were selected in heterospecific crosses between previously described (Tsygankov *et al.*, 1990a) donor '*M. flagellatum*' MFK65 and RecA⁻ Bio⁻ *E. coli* strains.

Interrupted matings. Wild-type '*M. flagellatum*' strains MFK64 (Serebrijski *et al.*, 1989) and MFK95 (Tsygankov *et al.*, 1990b)

carrying chromosomally-integrated plasmid pAS8-121 were used as donors. Interrupted matings were carried out on filters. Interruptions were performed at various times after mixing donor and recipient by vigorously vortexing for 1 min. The samples were incubated overnight in non-selective medium containing streptomycin (3.5 mg ml⁻¹) for donor counter-selection. The washed-off cultures were then plated onto selective media to determine the numbers of donor, recipient and recombinant cells (samples of known quantities of biotin were added to minimal liquid medium tubes seeded with *E. coli* strain R871, and the optical densities of the overnight cultures were compared).

Measurement of intracellular (free and bound) biotin. To extract free intracellular biotin, '*M. flagellatum*' cells (harvested from 1.5 ml of overnight culture) were washed once with phosphate buffer (50 mM, pH 6.8) and boiled in distilled water for 5 min. The biotin concentration of the supernatant was determined by microbiological assay. To extract bound biotin, washed cells were hydrolysed with 0.5 ml 3 M H₂SO₄ at 121 °C for 30 min. After the hydrolysate had been neutralized to pH 6.8 with NaOH, the biotin concentration was measured by microbiological assay.

RESULTS

'*M. flagellatum*' intracellular biotin content, biotin excretion and biotin degradation

Biotin can be synthesized, accumulated and excreted by some micro-organisms, while other species do not excrete it or are unable to synthesize the biotin required for their growth. Wild-type '*M. flagellatum*', a biotin prototroph, was tested in cross-feeding experiments with *E. coli* R874. The results showed that '*M. flagellatum*' did not excrete biotin or its biosynthetic intermediates in detectable amounts.

Intracellular biotin concentrations in cultures of '*M. flagellatum*' and (for comparison) of *E. coli* grown in the absence of exogenous biotin were examined. In the wild-type '*M. flagellatum*' strain MFK1, the majority of intracellular biotin was assayable only after acid hydrolysis. The total intracellular biotin content of the '*M. flagellatum*' cells was very similar to that of *E. coli* TG1 cells: about 4.5 ng biotin per mg cells (dry weight).

Some micro-organisms are known to degrade D-biotin to bisnorbiotin and tetranorbiotin via β -oxidation (Iwahara *et al.*, 1969). When '*M. flagellatum*' MFK1 was grown overnight in a minimal medium containing methanol in the presence of D-biotin at 1–10 $\mu\text{g l}^{-1}$, no decrease in its concentration was detectable, indicating a lack of biotin-degrading activity.

Isolation of mutants defective in biotin biosynthesis and determination of metabolic lesions in some of these mutants

Although the biotin requirement of different bacteria is as low as 5–50 $\mu\text{g l}^{-1}$ (Eisenberg, 1985), our attempts to select Bio⁻ mutants of '*M. flagellatum*' using this range of biotin concentrations were unsuccessful. Only after increasing the concentration of biotin about 10³-fold (up to 25 mg l⁻¹) did we succeed in isolation of 17 Bio⁻ mutants.

Using a medium containing dethiobiotin, a direct pre-

Table 1. Strains and plasmids used in this study

Strain	Relevant genotype/phenotype	Source/reference
<i>Escherichia coli</i>		
HFR KL-16	<i>recA56 stlC::Tn10</i>	Laboratory collection
R871	<i>bioB2</i>	Cleary & Campbell (1972)
R874	<i>bioF12</i>	Cleary & Campbell (1972)
R874r	<i>bioF12 recA56 stlC::Tn10</i>	This study
R877	<i>bioD19</i>	Cleary & Campbell (1972)
R878	<i>bioC23</i>	Cleary & Campbell (1972)
R878r	<i>bioC23 recA56 stlC::Tn10</i>	This study
R879	<i>bioA24</i>	Cleary & Campbell (1972)
BM355	<i>bioH::Mucts pro</i>	Barker & Campbell (1980)
AT2687	<i>bioA1 metB1</i>	Laboratory collection
AT2687r	<i>bioA1 metB1 recA56 stlC::Tn10</i>	This study
L37	<i>thi thr ilvC</i>	Laboratory collection
<i>'Methylobacillus flagellatum'</i> (laboratory collection)		
MFK3	<i>leu-1 str-1</i>	Tsygankov & Kazakova (1987)
MFK75	<i>leu-1 ilvA1 str-1 nal-7</i>	Nal ^R derivative of MFK19
MFK64	HfrA (Km ^R Tc ^R Sm ^R Tp ^R)	Serebrijski <i>et al.</i> (1989)
MFK65	Plasmid pULB113	Tsygankov <i>et al.</i> (1990a)
MFK77	<i>gen-1</i> , plasmid R68.45	Kletsova & Tsygankov (1990)
MFK95	HfrB (Km ^R Tc ^R Sm ^R Tp ^R)	Tsygankov <i>et al.</i> (1990b)
Biotin mutants of '<i>M. flagellatum</i>' obtained in this study		
MFK29-40	<i>leu-1 str-1</i> (common genotype)	NTG treatment of MFK3
MFK24	<i>bio-3</i>	
MFK25	<i>bioA2</i>	
MFK26	<i>bioB1</i>	
MFK27	<i>bio-4</i>	
MFK28	<i>bio-5</i>	
MFK33	<i>bioD1</i>	
MFK36	<i>bioA1</i>	
MFK29-32, 34, 35, 37-40	<i>bioB2:10</i>	
MFK60	<i>bioB1 rif-14 nal-4</i>	Rif ^R Nal ^R derivative of MFK26
MFK61	<i>bioA1 rif-15 nal-5</i>	Rif ^R Nal ^R derivative of MFK36
MFK85-94	<i>leu-1 str-1 ilvA1 nal-7</i> (common genotype)	NTG treatment of MFK75
MFK85	<i>bioD2</i>	
MFK86	<i>bioA3</i>	
MFK87	<i>bioA4</i>	
MFK88-90	<i>bioD3:5</i>	
MFK91-94	<i>bio-6:9</i>	
Plasmids		
pULB113	IncP1, Km ^R Tc ^R Ap ^R	Lejeune <i>et al.</i> (1983)
R68.45	IncP1, Km ^R Tc ^R Ap ^R	Haas & Holloway (1976)

cursor of biotin, we selected 10 other mutants. The frequency of mutant isolation was 0.54 % on biotin and 0.1 % on dethiobiotin. The level of reversions to the wild phenotype for all *bio* mutations did not exceed 10⁻⁸ per cell. Three of the mutants demonstrated a leaky phenotype. The minimal requirements of the mutants for biotin

and dethiobiotin were 8 and 4 mg l⁻¹, respectively. Some of the mutants were able to grow in the presence of either biotin or dethiobiotin, while others grew only in the presence of biotin. The ability of mutants to be complemented only by biotin indicates a lesion in *bioB*, responsible for synthesis of biotin synthetase.

Table 2. Analysis of the '*M. flagellatum*' Bio⁻ mutants in cross-feeding experiments with known *bio* mutants of *E. coli*

	' <i>M. flagellatum</i> ' mutants:			
	MFK75	29-32, 34, 35, 37-40	61, 80, 85-90	82-84, 91-94
Fed by:				
Biotin	<i>bio</i> ⁺	+	+	+
Dethiobiotin	<i>bio</i> ⁺	—	+	+
Pimelate	<i>bio</i> ⁺	—	—	—
Feeding of <i>E. coli</i> mutants				
<i>bioB</i>	—	—	—	—
<i>bioD</i> , <i>bioA</i>	—	+	—	—
<i>bioC</i> , <i>bioF</i> , <i>bioH</i>	—	+	+	—
Classified as		<i>bioB</i>	<i>bioD</i> , <i>bioA</i>	Defective in some early steps

Neutralized pimelic acid (0.5-50 µg ml⁻¹) was used as a supplement in order to detect mutants dependent on pimelate for their biotin prototrophy; none of the mutants among our collection showed a strict requirement for this compound.

The amount of biotin biosynthetic intermediates excreted into the growth medium was insufficient to cross-feed the '*M. flagellatum*' mutants. In order to establish the metabolic blocks in the rest of the mutants, we carried out cross-feeding experiments using known *bio* mutants of *E. coli*. The results of these experiments are shown in Table 2.

The results of cross-feeding experiments using supernatants did not allow us to distinguish between *bioA* and *bioD* mutants. This may be because the amount of 7,8-diaminopelargonic acid excreted by *bioD* mutants of '*M. flagellatum*' is not sufficient to feed a *bioA* mutant of *E. coli*, while the amount of 7-keto-8-aminopelargonic acid excreted by '*M. flagellatum*' cells at the same time is sufficient to feed the mutants of *E. coli* defective in the earlier stages of biotin biosynthesis (*bioF, C, H*).

The addition of pimelic acid to the culture medium of several different micro-organisms has been shown to enhance the biosynthesis of biotin and its intermediates (Izumi *et al.*, 1980). Interestingly, no increase in excretion of biotin or its intermediates was observed after the addition of pimelate (0.5-50 mg l⁻¹) to '*M. flagellatum*' cultures (tested in a cross-feeding experiment with *E. coli* R874; data not shown). The experiments described above made it possible to divide all the *bio* mutants of '*M. flagellatum*' into three large groups.

Cloning of some of the *bio* genes of '*M. flagellatum*' in R-prime plasmids

A library consisting of 253 R-prime plasmids (Tsygankov *et al.*, 1990a) carrying various fragments of the '*M. flagellatum*' chromosome contained R-prime plasmids able to complement 33 auxotrophic mutations of *E. coli*, 17 of

'*M. flagellatum*' and 3 of *Pseudomonas aeruginosa*. R'*proA* plasmids selected using *E. coli* AB2463 complemented mutations *bioB1-11* in '*M. flagellatum*' and *bioB2* in *E. coli* R871. These plasmids also complemented *bioD* and *bioH* mutations in *E. coli* R877 and BM355 and six mutations of '*M. flagellatum*' (see Table 3), but did not complement any other *bio* mutations in the *E. coli* strains tested.

None of the R-prime plasmids in our library were able to complement *bioA, F, C* mutations in *E. coli*, or some unidentified *bio* mutations in '*M. flagellatum*', which indicated that the range of R-prime plasmids available was probably insufficient for complementation of all *bio* mutations of '*M. flagellatum*'. We therefore attempted to obtain R-prime plasmids carrying *bioA, bioF* and *bioC* by separate selection using complementation of corresponding recipient strains of *E. coli*.

Our preliminary data on constructing R-prime plasmids carrying fragments of the '*M. flagellatum*' chromosome indicated their instability in *recA*⁺ *E. coli* hosts. In order to obtain stable R-prime plasmids we constructed *recA*⁻ derivatives of *E. coli* AT2687 (*bioA1*), R874 (*bioF12*) and R878 (*bioC23*). A previously described donor strain of '*M. flagellatum*' was employed in heterospecific crosses for selection of R-prime plasmids.

Five R'*bioA* plasmids were selected by complementation of the *bioA1* mutation in recipient strain AT2687, and their ability to transfer the *bioA* marker into a Rif^R derivative of the same strain was tested. All five clones showed the Km^R Ap^R phenotype determined by the plasmid antibiotic resistance genes. All R'*bioA* plasmids also complemented the *bioA24* mutation in *E. coli* R879 (and four mutations of '*M. flagellatum*'; see Table 3), but none of the other *bio* mutations tested. Stable R-prime plasmids were not found among the transconjugants obtained in crosses with *bioF* and *bioC* mutants of *E. coli*. These results suggested that at least four genes involved in biotin biosynthesis in '*M. flagellatum*' had been cloned on R-prime plasmids.

Table 3. Classification of the Bio⁻ mutants of '*M. flagellatum*'

Characteristics of mutants	Mutants in groups I-V:				
	I	II	III	IV	V
	29-32, 34, 35, 37-40	80, 85, 88-90	82, 83, 93, 94	61, 81, 86, 87	84, 91, 92
Growth on dethiobiotin	-	+	+	+	+
Complemented with R' <i>proA</i>	+	+	+	-	-
Complemented with R' <i>bioA</i>	-	-	-	+	-
Feeds <i>E. coli bioF</i>	+	+	-	+	-
Identified as:	<i>bioB</i>	<i>bioD</i>	<i>bioH</i> (or/ and <i>bioC</i> , <i>bioF</i>)	<i>bioA</i>	<i>bioC</i> or/ and <i>bioF</i>

Classification of the *bio* mutations of '*M. flagellatum*'

In order to classify the Bio⁻ mutants of '*M. flagellatum*' we complemented them with the R-prime plasmids described above. One group of mutants was complemented by R'*proA*, the other by R'*bioA*. Some mutants were not complemented by any of the R-prime plasmids. No mutants could be complemented by both kinds of R-prime plasmids. Based on the complementation data and feeding tests, all Bio⁻ mutants could be divided into five groups (Table 3).

It is important to note that while groups I, II and IV are identified unequivocally, group III can be identified as *bioH* only if we assume that all the '*M. flagellatum*' genes carried by R-prime plasmids can be expressed effectively in *E. coli*. Otherwise, *bioC* or *bioF* mutants could also be present in group III. Although mutants belonging to groups III and V show different patterns of complementation by R-prime plasmids, cloning of individual '*M. flagellatum*' genes is needed to classify the mutations precisely.

Mapping of *bio* mutations of '*M. flagellatum*'

Complementation mapping of *bioB, D, H* mutations of '*M. flagellatum*'. All 29 R'*proA* plasmids complemented the *bioB2*, *bioD19* and *bioH* mutations in corresponding *E. coli* strains; 16 of them also complemented the *argE3* mutation in *E. coli* AB2463. Seven of 47 R'*argE* plasmids complemented the *proA2* mutation, but none complemented *bioB* mutations. It could be concluded that '*M. flagellatum*' genes complementing these mutations are linked and their order is *CargE3-CproA2-bioB1* ['*M. flagellatum*' genes identified by complementation of *E. coli* strains, according to Lyon *et al.* (1988), are indicated by the prefix C]. The established order was confirmed by the results of analysis of linkage with marker *ilvC* in strain L37. One R'*proA* plasmid, but none of the R'*argE* plasmids complemented the mutation, which places *ilvC* next to *bioB*, making the most likely gene order as follows: *CargE3-CproA2-bioB-CilvC*. The data obtained allow us to place three of

the '*M. flagellatum*' *bio* genes mentioned above into a large group of 15 auxotrophic genes determined earlier (Tsygankov *et al.*, 1990a) (see Fig. 2).

Mapping of the *bioA* mutation in '*M. flagellatum*'. The R'*bioA* plasmids were further tested for the ability to complement various mutations in *E. coli* and '*M. flagellatum*' strains defective in the biosynthesis of amino acids, vitamins and nucleotides. No linkage was revealed with 38 different markers tested. Restriction analysis of R-prime plasmids has shown that they carried inserts of about 15 kb (data not shown).

For chromosomal mapping of various Gram-negative bacteria the IncPI plasmids are often used; these mediate unorientated chromosome mobilization and thus allow determination of the cotransfer frequencies of different markers. Although the determination of true marker linkage values can be hampered by retroconjugant formation (Mergeay *et al.*, 1987), a system of chromosome mobilization with the aid of the IncP1 plasmid R68.45 was successfully applied for mapping of methylotrophs. In homologous matings the linkage of C-1 genes of *Methylobacterium* AM1 (Tatra & Goodwin, 1985) and of '*M. flagellatum*' (Kletsova & Tsygankov, 1990) with different antibiotic resistance and auxotrophic markers was determined.

We employed the system of chromosome mobilization using R68.45 to determine the linkage of '*M. flagellatum*' *bioA* markers with *ilvA1* and *leu-1*, which belong to the early and late chromosome regions, respectively (Serebrijski *et al.*, 1989). Analysis of recombinant classes, obtained in the matings described earlier (Kletsova & Tsygankov, 1990), has shown that the *leu-1* marker (and a group of nine other linked markers) is not linked to *bioA1*. These data were confirmed by the results of analysis of cotransfer frequencies of unselected markers in matings between wild-type donor (MFK77) and mutant MFK86. No linkage was found between *bioA2* and *ilvA2* (data not shown).

The results of '*M. flagellatum*' chromosome mapping (Tsygankov *et al.*, 1990a) are consistent with the idea of

obligate methylotroph ('*M. flagellatum*'). Our current data allowed us to put five additional (*bio*) markers on this map. Our data demonstrate that, unlike in *E. coli*, the *bioA* gene is not linked to the *bioB* and *bioD* genes in '*M. flagellatum*'. Conversely, while in *E. coli* *bioH* is located far from the *bioA-D* operon, it is closely linked to the *bioB* and *bioD* genes in '*M. flagellatum*'. The location of other '*M. flagellatum*' *bio* genes is still unknown. At least four *bio* genes are clustered on the '*M. flagellatum*' chromosome (*bioBDH* genes and one of the genes of early stages of biotin biosynthesis). Whether the genes are organized in an operon structure has yet to be determined. Cloning and study of individual genes, which is currently in progress, may help to clarify this.

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REFERENCES

- Barker, D. F. & Campbell, A. M. (1980). Use of *bio-lac* fusion strains to study regulation of biotin biosynthesis in *Escherichia coli*. *J Bacteriol* **143**, 789–800.
- Cleary, P. & Campbell, A. M. (1972). Deletion and complementation analysis of the biotin gene cluster of *Escherichia coli*. *J Bacteriol* **112**, 830–839.
- Eisenberg, M. A. (1985). Regulation of the biotin operon in *E. coli*. Part IV. Biotin synthesis and metabolism, biotin as molecular probe. *Ann NY Acad Sci* **447**, 335–349.
- Eisenberg, M. A. (1987). Biosynthesis of biotin and lipoic acid. In *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology*, pp. 544–550. Edited by F. C. Neidhart, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter & M. E. Umberger. Washington, DC: American Society for Microbiology.
- Gloeckler, R., Ohsawa, I., Speck, D., Ledoux, C., Bernard, S., Zinsius, M., Villeval, D., Kisou, T., Komogawa, K. & Lemoine, U. (1990). Cloning and characterization of the *Bacillus sphaericus* genes controlling the bioconversion of pimelate into dethiobiotin. *Gene* **87**, 63–70.
- Gomelsky, M., Gak, E., Chistoserdov, A., Bolotin, A. & Tsygankov, Y. D. (1990). Cloning, sequence and expression in *Escherichia coli* of the *Methylobacillus flagellatum* *recA* gene. *Gene* **94**, 69–75.
- Haas, D. & Holloway, B. W. (1976). R factor variants with enhanced sex factor activity in *Pseudomonas aeruginosa*. *Mol & Gen Genet* **144**, 243–251.
- Holloway, B. W. (1984). Genetics of methylotrophs. In *Methylotrophs: Microbiology, Biochemistry, and Genetics*, pp. 87–106. Edited by C. Hou. Boca Raton, FL: CRC Press.
- Holloway, B. W. & Morgan, A. F. (1986). Genome organization in *Pseudomonas*. *Annu Rev Microbiol* **40**, 79–105.
- Iwahara, S., McCormick, D. B., Wright, L. D. & Li, H. C. (1969). Bacterial degradation of biotin. *J Biol Chem* **244**, 1393–1398.
- Izumi, Y., Tani, Y. & Yamada, H. (1980). Biotin biosynthesis in microorganisms. *Bull Inst Chem Res Kyoto Univ* **58**, 434–447.
- Kiriukhin, M. Y., Detkov, S. Y., Baev, M. V. & Tsygankov, Y. D. (1993). Citrate synthase from the obligate methylotroph *Methylobacillus flagellatum*. *FEMS Microbiol Lett* **113**, 101–106.
- Kletsova, L. V. & Tsygankov, Y. D. (1990). Mapping of *pgi* and *gpd* genes involved in C-1 assimilation in the obligate methylotroph *Methylobacillus flagellatum*. *Arch Microbiol* **153**, 139–145.
- Lejeune, P., Mergeay, M., van Gijsegem, F., Faelen, M., Certis, J. & Toussaint, A. (1983). Chromosome transfer and R-prime plasmid formation mediated by plasmid pULB113 (RP4::mini-Mu) in *Alcaligenes eutrophus* CH34 and *P. fluorescens* 6.2. *J Bacteriol* **155**, 1015–1026.
- Lyon, B. R., Kearney, P. P., Sinclair, M. I. & Holloway, B. W. (1988). Comparative complementation mapping of *Methylophilus* spp. using cosmid gene libraries and prime plasmids. *J Gen Microbiol* **134**, 123–132.
- Mergeay, M., Lejeune, P., Sadouk, A., Gerits, J. & Fabry, L. (1987). Shuttle transfer (or retrotransfer) of chromosomal markers mediated by plasmid pULB113. *Mol & Gen Genet* **209**, 61–70.
- Moore, A. T., Nayudu, M. & Holloway, B. W. (1983). Genetic mapping in *Methylophilus methylotrophus* AS1. *J Gen Microbiol* **129**, 785–799.
- Pai, C. H. (1975). Genetics of biotin synthesis in *Bacillus subtilis*. *J Bacteriol* **121**, 1–8.
- Rolfe, B. & Eisenberg, M. A. (1968). Genetic and biochemical analysis of the biotin loci of *Escherichia coli* K-12. *J Bacteriol* **96**, 515–524.
- Sakurai, N., Imai, Y. J., Masuda, M., Komatsubara, S. & Tosa, T. (1993). Molecular breeding of a biotin-hyperproducing *Serratia marcescens* strain. *Appl Environ Microbiol* **59**, 3225–3232.
- Serebrijski, I. G., Kazakova, S. M. & Tsygankov, Y. D. (1989). Construction of Hfr-like donors of the obligate methanol-oxidizing bacterium *Methylobacillus flagellatum* KT. *FEMS Microbiol Lett* **59**, 203–206.
- Tatra, P. K. & Goodwin, P. M. (1985). Mapping of some genes involved in C-1 metabolism in the facultative methylotroph *Methylobacterium* sp. strain AM1 (*Pseudomonas* AM1). *Arch Microbiol* **143**, 169–177.
- Tsygankov, Y. D. & Kazakova, S. M. (1987). Development of gene transfer systems in *Methylobacillus flagellatum* KT: isolation of auxotrophic mutants. *Arch Microbiol* **149**, 396–401.
- Tsygankov, Y. D., Kazakova, S. & Serebrijski, I. G. (1990a). Genetic mapping of the obligate methylotroph *Methylobacillus flagellatum*: characteristics of prime plasmids and the chromosome map in time units. *J Bacteriol* **172**, 2747–2754.
- Tsygankov, Y. D., Gomelski, M. V., Marchenko, G. N. & Serebrijski, I. G. (1990b). Genetics of methylotrophs. In *Proceedings, 6th International Symposium on Genetics of Industrial Microorganisms*, pp. 645–656. Edited by H. Heslot and others. Strasbourg: Société Française de Microbiologie.

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