JOURNAL OF BACTERIOLOGY, Oct. 1973, p. 331-336
Vol. 116, No. 1
Copyright © 1973 American Society for Microbiology
Printed in U.S.A.

Pyrimidine Metabolism in *Acinetobacter calcoaceticus*

STEINAR ØVREBØ AND KJELL KLEPPE
Department of Biochemistry, University of Bergen, Bergen, Norway

Received for publication 2 July 1973

The metabolism of thymine, thymidine, uracil, and uridine has been investigated in five different strains of *Acinetobacter calcoaceticus*. Attempts to isolate thymine and thymidine auxotrophic mutants were not successful. Consistent with this finding was the observation that uptake of radioactive thymine or thymidine could not be demonstrated. Search for enzymes capable of transforming thymine via thymidine to thymidine-5′-monophosphate in crude extracts was performed, and the following enzymes were absent judging from enzyme assays: thymidine phosphorylase (EC 2.4.2.4), trans-*N*-deoxyribosylase (EC 2.4.2.6), and thymidine kinase (EC 2.7.1.21). The enzymes responsible for the phosphorylation of thymidine-5′-monophosphate to thymidine-5′-triphosphate were present in crude extracts. Radioactive uracil was readily incorporated into both ribonucleic acid and deoxyribonucleic acid, the ratio being 6:1, and radioactivity was found only in pyrimidine bases. No uptake of uridine could be demonstrated. Uridine-5′-monophosphate pyrophosphorylase (EC 2.4.2.9) activity was detected in crude extracts, suggesting that uracil is converted directly to uridine-5′-monophosphate which is then phosphorylated to uridine-5′-triphosphate or transformed to other ribo- and deoxypyrimidine nucleotides.

The mechanisms of synthesis of nucleic acids are currently under intense investigation. In bacteria as well as higher organisms it is realized that these mechanisms may vary for different organisms. Most of our knowledge concerning the synthesis of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) in bacteria has been obtained from studies on *Escherichia coli* and phage-infected *E. coli*. However, to make any generalizations with regard to nucleic acid synthesis in bacteria, it is necessary to obtain information from a wide variety of different microorganisms.

In this laboratory we have recently embarked on a study of the mechanisms of DNA and RNA synthesis in *Acinetobacter calcoaceticus*. This is a widely occuring species which differs from *E. coli* in many respects (22). A number of reports have appeared in the last years both on metabolism (23, 24) and taxonomy (1, 9, 29, 22) of this species.

The synthesis of nucleic acids in most organisms is conveniently followed by measuring the incorporation of radioactivity from radioactive precursors. It is therefore essential to have a good knowledge of the various metabolic pathways for the nucleic acid precursors. In the present work we describe the metabolism of uracil, uridine, thymine, and thymidine in *A. calcoaceticus*.

MATERIALS AND METHODS

Bacterial strains. The main strain used in this work was *A. calcoaceticus* NCTC 7363. Enzymatic analyses were also carried out on the following strains of *A. calcoaceticus:* ATCC 19606, ATCC 9956, ATCC 11959, and a clinical isolate, 62819, made available by Department of Microbiology, Copenhagen, originally obtained from R. B. Lindberg, U.S.A. *E. coli* B wild type was from the laboratory stock (originally it was a gift from O. Sköld, Uppsala, Sweden).

Chemicals. Thymine, thymidine, uracil, uridine, thymidine mono-, di-, and tri-phosphates (TMP, TDP, and TTP, respectively), adenosine triphosphate (ATP) and salmon sperm DNA were from the Sigma Chemical Co., U.S.A. 5-Fluoro-uracil and 5-fluoro-deoxyuridine were gifts from Hoffmann-La Roche, Switzerland. Trimethoprim [2,4-diamino-5(3′,4′,5′-trimethoxybenzyl)pyrimidine] was from Burroughs Wellcome & Co., London. Aminopterin was a product of Schuchardt Munic, Germany, and carbenicillin was from Astra, Sweden. ^{14}C-thymine (thymine-2-^{14}*C*, 58.3 mCi/mmol), ^{3}H-thymidine (thymidine-*methyl*-^{3}*H*, 25 Ci/mmol), ^{14}C-uracil (uracil-2-^{14}*C*, 61 mCi/mmol), ^{3}H-uridine (uridine-5,6-^{3}*H*, 58 Ci/mmol) were purchased from the Radiochemical Centre, Amersham, England. ^{3}H-TMP (*methyl*-^{3}*H*) were prepared by the method of Okazaki and Kornberg (19). All radiochemicals were

checked for purity by paper chromatographic methods.

Media. The minimal medium used contained the following compounds per liter: NaCl, 0.58 g; $MgSO_4$, 0.12 g; $CaCl_2$, 0.011 g; $FeCl_3$, 0.16 mg; NH_4Cl, 1 g; KH_2PO_4, 3 g; $Na_2HPO_4 \cdot 2H_2O$, 7.8 g. For all strains except NCTC 7363 20 ml of Hutner base (5) per liter was also added. The carbon source in case of *A. calcoaceticus* was 0.2% acetate and for *E. coli* B 0.2% glucose. The rich medium occasionally used contained per liter: yeast extract (Difco), 8 g; and NaCl, 5 g. The carbon sources were as above.

Measurement of growth and incorporation of radioactivity. Growth was followed by measuring the absorbance at 450 nm in a Zeiss spectrophotometer PMQII. Samples of 1 ml were withdrawn and pipetted into 50 μliters of 20% Formalin to stop growth. An absorbtion of 1 at 450 nm corresponds to 8×10^8 cells/ml.

Incorporation of radioactivity was determined by collecting material insoluble in 5% trichloroacetic acid on Satorius nitro-cellulose membrane filters. The dried filters were then subsequently counted in a liquid scintillation counter. For incorporation of radioactivity into DNA with radioactively labeled uracil as a precursor, the cells were first subjected to hydrolysis in 0.3 M KOH for 16 h at 37 C, and then the material was precipitated in 5% trichloroacetic acid and counted as described above.

Mutagenesis. For selection of thymine-requiring mutants the method of Smith (21) employing aminopterin was used. The cells were pretreated with and without ethylenediaminetetraacetic acid (EDTA) in phosphate buffers at various temperatures, and the screening of mutants on agar plates was performed with concentration of thymine up to 80 μg/ml. Selection of thymidine-requiring mutants was carried out by the method of Dale and Greenberg (6). The concentration of trimethoprim was 16 μg/ml and that of thymidine 0.5 mg/ml. For auxotophic mutants other than thymine and thymidine, the penicillin selection method was employed (7, 12). Carbenicillin was used to concentrate auxotrophic mutants.

Thymidine phosphorylase and trans-N-deoxyribosylase. The cells were grown until the absorbance at 450 nm was 1, then washed in 0.05 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 7.0) and suspended in ¼ the original volume in the same buffer. A French pressure cell was used to open the cells at a pressure of 24,000 lb/in^2. Occasionally whole cells were also used. The assays for these enzymes were the same as those described by Munch-Pettersen (18).

Thymidine kinase. Exponentially grown bacteria were harvested when the absorbance at 450 nm had reached 1, washed in 0.9% NaCl, and suspended in a volume 1/100 the original in 0.1 M Tris-hydrochloride (pH 7.8)-10 mM $MgCl_2$ - 2 mM 2-mercaptoethanol.

This suspension was then subjected to French pressure cell treatment. Part of the crude extract was centrifuged at 17,000 × *g* for 15 min. Two different assay methods were employed for thymidine kinase, namely that of Beck et al. (2) and the procedure of Okazaki and Kornberg (19). With both procedures the concentration of ATP used was 7 mM, whereas the concentration of thymidine was 0.05 mM and 0.84 mM, respectively. Time of incubation was 60 min at 37 C.

TMP and TDP kinase. The cells were grown and opened as described above for thymidine kinase. The reaction mixture contained 0.1 M Tris-hydrochloride (pH 7.8), 7 mM ATP, 10 mM $MgCl_2$, 2 mM 2-mercaptoethanol, and 0.5 mM ^{3}H-TMP, specific activity 0.77 mCi/mmol. The volume was 100 μliters and reaction temperature was 37 C. Samples of 10 μliters were withdrawn at various times and spotted on PEI cellulose plates which were developed in 1 M LiCl. The spots corresponding to TMP, TDP, and TTP were cut out and counted in a liquid scintillation counter.

Phosphatase and 5'-nucleotidase. The reaction mixture contained the same components as given for thymidine kinase by Beck et al. (2), except that ^{3}H-TMP (0.5 mM, specific activity 0.77 mCi/mmol) was substituted for thymidine. Some experiments were also conducted in the absence of ATP.

Uridine monophosphate (UMP)-pyrophosphorylase and uridine phosphorylase. Growth of cells and opening of these were as described for thymidine kinase except that the buffer consisted of 0.05 M Tris-hydrochloride (pH 7.3), 10 mM $MgCl_2$, and 2 mM mercaptoethanol. The assays and separation of product were as described by Beck et al. (2).

Uridine kinase. The preparation of extracts was as described for thymidine kinase. The assay was carried out according to Beck et al. (2).

Isolation of DNA. Two different methods for DNA isolation were employed.

(i) DNA for T_m determination. DNA for melting temperature (T_m) determination was isolated by the method of DeLey (8). The concentration of DNA was determined by the diphenylamine test by the method of Burton (4) or by absorbance at 260 nm.

(ii) Radioactive DNA for base analysis. Approximately 25 mg of ^{14}C-uracil-labeled cells was suspended in 300 μliters of a solution consisting of 0.15 M NaCl and 0.1 M EDTA. Then 15 μliters of 25% sodium dodecyl sulfate was added, and the suspension was incubated for 10 min at 60 C. The mixture was then diluted to 2 ml with the same buffer as above and extracted twice with phenol saturated with 0.1 × SSC (0.15 M NaCl, 0.015 M trisodium citrate, pH 7.2 ± 0.2). The phenol phases were combined and extracted with 1 × SSC. The two water phases were combined, and the nucleic acids were precipitated by addition of two volumes of ethanol. The precipitate was resuspended in 0.1 × SSC and with 0.3 M KOH and incubated for 16 h at 37 C. The solution was next dialyzed against 0.1 × SSC for 24 h, 2.5 mg of salmon sperm DNA was added as carrier, and the solution made 1 × SSC. The DNA was then precipitated by addition of two volumes of ethanol. This precipitate was washed in 70% ethanol and dried. Base hydrolysis by $HClO_4$ was performed by the method of Marshak and Vogel (17). The bases were subjected to descending paper chromatography on Whatmann 1 MM paper in the following solvent system: solvent system I—2 N HCl in isopropanol-water (65:35); solvent

system II—1 n-butanol-0.1 N NH_3 (6:1) (3).

Determination of GC content. The guanine and cytosine (GC) content was determined by two methods.

(i) By T_m. The procedure of Marmur and Doty was employed (16). DNA from *E. coli* B was used as a control for the system.

(ii) By bouyant density determination in CsCl. This was accomplished by use of equilibrium centrifugation in analytical ultracentrifuge, Spinco model E. The centrifuge was run at 44,770 rpm at 20 C for at least 20 h with a 2° 12-mm Kel-F centerpiece. The photographic pictures were scanned in a Schimadzu MPS-50L spectrophotometer, and the GC content was determined by the method of Mandel et al. (15).

Determination of proteins. Protein was determined by the method of Lowry et al. (13).

RESULTS

Five different strains of *A. calcoaceticus* were employed in the present work. The mutation experiments and studies on incorporation of radioactive bases and nucleosides were performed only with strain NCTC 7363. All enzymatic analyses were, however, carried out with all five strains. Since the results were identical for all strains, we list only the data obtained with strain NCTC 7363.

Metabolism of thymine and thymidine. DNA synthesis in many organisms is conveniently followed by measuring the incorporation of thymine or thymidine. For this purpose, thymine and thymidine auxotrophic mutants are employed. A number of attempts were made to isolate such mutants in *A. calcoaceticus* using the folic acid antagonists aminopterin and trimethoprim, and thymime and thymidine concentrations up to 80 and 500 μg/ml, respectively. No thymine or thymidine mutants were, however, obtained. These results suggested that *A. calcoaceticus* is unable to utilize both thymine and thymidine. Consequently, neither radioactive thymine or thymidine would be expected to be incorporated into DNA in this organism. No incorporation of ^{14}C-thymine was observed, whereas for *E. coli* B, the control, the incorporation was as expected. Similar results were also obtained with ^{3}H-thymidine. In the case of thymine, various concentrations of deoxyadenosine up to 2 mM were employed. Experiments were also conducted with the other deoxynucleotides as well. No incorporation was, however, observed.

A prerequisite for the isolation of thymine mutants is the existence of enzymes capable of converting thymine to thymidine monophosphate. The results presented above suggested that these enzymes are not present in *A. calcoaceticus*. Extracts from all five strains were made, and the activity of various metabolic enzymes was tested. Again the control was *E. coli* B.

The results for the enzymes thymidine phosphorylase (EC 2.4.2.4; reaction 1, Fig. 5) and *trans-N*-deoxyribosylase (EC 2.4.2.6; reaction 1, Fig. 5) are given in Table 1. No activity was observed in the extracts from *A. calcoaceticus* for these enzymes. Both enzymes were present in extracts from *E. coli* B. Similar results were obtained with the enzyme thymidine kinase (EC 2.7.1.21; reaction 2, Fig. 5). The absence of the latter enzyme in extracts from *A. calcoaceticus* was not due to the presence of 5′-nucleotidase or phosphatase activity. Several attempts were made to induce the above mentioned enzymes by growing the cells in minimal acetate medium in the presence of thymine or thymidine. However, no enzymatic activity was found in the extracts from these cells.

Extracts from *A. calcoaceticus* did contain enzymes which converted TMP to TDP and TTP (reaction 3 and 4, Fig. 5; Table 2). These appeared to be present in approximately the same amount as in *E. coli* B.

Metabolism of uracil and uridine. The effect of the growth inhibitors (2) 5-fluorouracil and 5-fluorodeoxyuridine on *A. calcoaceticus* is shown in Fig. 1. Only 5-fluorouracil inhibited growth, indicating that uracil is metabolized whereas 5-fluorodeoxyuridine, as expected, is not. From strain NCTC 7363 a uracil mutant was isolated by the penicillin selection method. This mutant was unable to utilize uridine,

TABLE 1. *Activity of thymidine phosphorylase and trans-N-deoxyribosylase in A. calcoaceticus and E. coli B*

Organism	Thymidine phosphorylase[a]	*Trans-N*-deoxyribosylase[a]
E. coli B	73.8	11.6
A. calcoaceticus	<1.3	<1.3

[a] The activity is expressed as nanomoles per milligram of protein per minute.

TABLE 2. *Phosphorylation of dTMP in extracts from A. calcoaceticus and E. coli B*

Organism	Nucleotides formed[a] (nmol)	
	dTDP	dTTP
E. coli B	0.66	0.50
A. calcoaceticus	0.33	0.30

[a] The numbers given are nanomoles formed per 30 min using a protein concentration of 2.4 mg per ml.

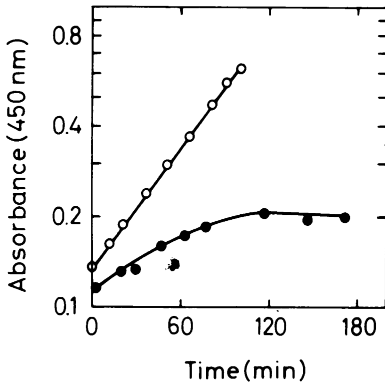

FIG. 1. *Effect of 5-fluorodeoxyuridine* (○) *concentration (250 μg/ml) and 5-fluorouracil* (●) *concentration (0.5 μg/ml) on the growth of A. calcoaceticus strain NCTC 7363.*

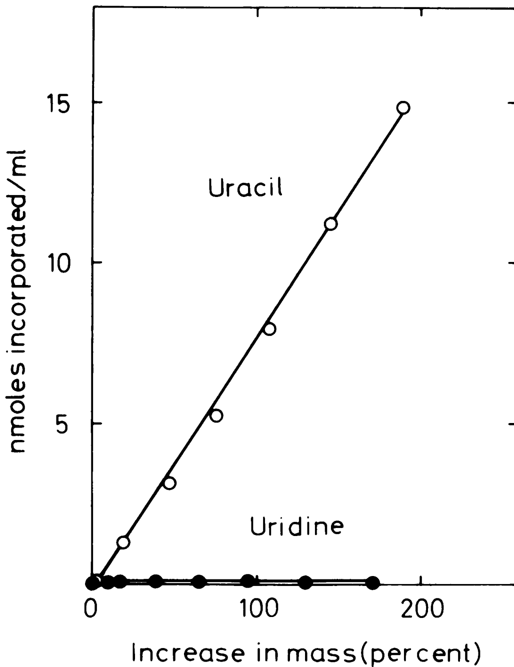

FIG. 2. *Incorporation of ^{14}C-uracil and ^{3}H-uridine in A. calcoaceticus strain NCTC 7363. The concentration and specific activity were 1.6 μg/ml (1.0 μCi/μg) and 2 μg/ml (0.1 μCi/μg), respectively. Incorporation was started when the absorbance at 450 nm had reached 0.2.*

suggesting that uridine is not metabolized in *A. calcoaceticus*. Futher evidence for this view is given in Fig. 2 which shows the incorporation of radioactive uracil and uridine. Uracil was readily taken up by the cells, no uridine was incorporated, indicating that uridine kinase (EC 2.7.1.48; reaction 7, Fig. 5) and uridine phosphorylase (EC 2.4.2.3; reaction 5, Fig. 5) are not present in *A. calcoaceticus*. No enzymatic activity corresponding to these enzymes was found in crude extracts, confirming the above hypothesis. Normal amounts of the enzyme UMP pyrophosphorylase (EC 2.4.2.9, reaction 6, Fig. 5) were found in extracts from this bacterium.

Labeling pattern of RNA and DNA. Radioactivity was observed both in RNA and DNA when ^{14}C-uracil was supplied to a culture of exponentially growing *A. calcoaceticus* (Fig. 3). The ratio between RNA and DNA, approximately 6 : 1, was the same as previously reported for *E. coli* under similar growth conditions (14). Both RNA and DNA which had been labeled for several generations were isolated and degraded to bases by acid treatment as described above. The bases were then separated by paper chromatography. In the case of RNA, radioactivity was observed in uracil and cytosine, and for DNA it was observed in thymine and cytosine. The separation pattern in one solvent system for the bases obtained from hydrolysis of DNA is given in Fig. 4. The ratio of counts between cytosine and thymine plus cytosine was found to be 39.9% which is in excellent agreement with GC content determined for this strain by physical methods. The GC content for strain NCTC

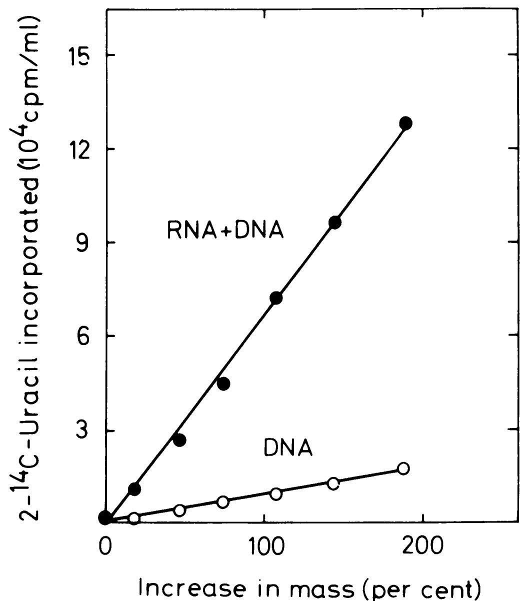

FIG. 3. *Incorporation of ^{14}C-uracil into RNA and DNA in A. calcoaceticus strain NCTC 7363. The concentration of uracil was 1.6 μg/ml and the specific activity 0.05 μCi/μg. Samples of 0.1 ml were withdrawn for measurement of trichloroacetic acid-isoluble radioactivity* (●) *and for alkali-stable trichloroacetic acid-insoluble radioactivity* (○).

7363 calculated from T_m measurement and CsCl bouyant density gradient centrifugations was 40.3 and 40.8%, respectively. These results indicate that uracil is converted very efficiently to cytosine and thymine nucleotides.

DISCUSSION

The metabolism of pyrimidines in some enteric bacteria is now well established (2). The pathways for uracil and thymine in *E. coli* and *Salmonella thyphimurium* are shown in Fig. 5. Exogenous uracil, uridine, thymine, and thymidine can be metabolized by these organisms. The results presented in the present work show that in the case of *A. calcoaceticus* only uracil of the above mentioned compounds is metabolized. The enzymes thymidine phosphorlase (reaction 1, Fig. 5), *trans-N*-deoxyribosylase (reaction 1), thymidine kinase (reaction 2), uridine kinase (reaction 7) and uridine phosphorylase (reaction 5) are not present in *A. calcoaceticus*. The pathway for utilization of exogeneous uracil in this organism can be given as shown in Fig. 6. Uracil can therefore be used to label both RNA and DNA in *A. calcoaceticus*. No attempts were made in the present work to study the mechanisms of conversion of uridine ribonucleotides to deoxynucleotides.

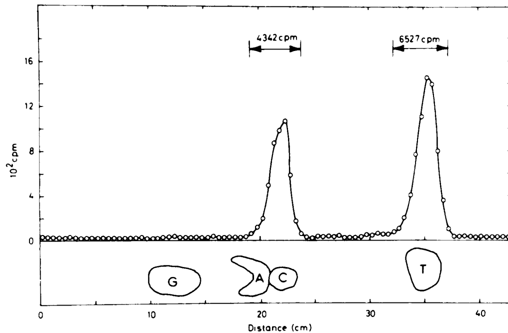

FIG. 4. *Paper chromatographic separation of percloric acid hydrolysate of* [14]*C-uracil-labeled DNA from strain NCTC 7363. Solvent system I was used (see Materials and Methods). The chromatogram was cut into pieces of 0.5 cm which were counted in a liquid scintillation counter.*

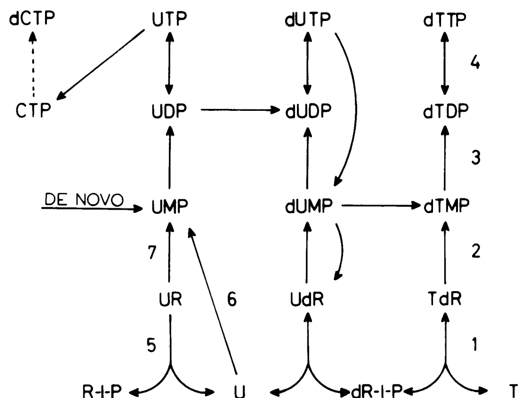

FIG. 5. *Metabolism of uracil and thymine in E. coli and Salmonella typhimurium.*

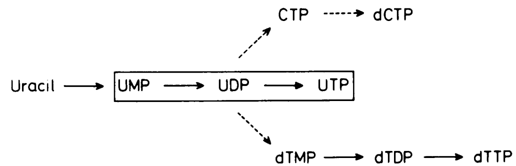

FIG. 6. *Metabolism of exogeneous uracil in A. calcoaceticus. Broken arrows are meant to represent several enzymatic reactions.*

The absence of metabolizing enzymes for thymine and thymidine in *A. calcoaceticus* is analogous to that observed in *Neisseria meningitides* (10, 11), a bacterium which is closely related to *A. calcoaceticus*. It is possible, therefore, that uptake of thymine or assay for an enzyme such as thymidine kinase could be a valuable tool in taxonomic studies of these microorganisms.

ACKNOWLEDGMENTS

This investigation was supported by grants from The Nansen Foundation and from The Norwegian Research Council.

We are grateful for help and advice from Per E. Joner.

LITERATURE CITED

1. Baumann, P., M. Doudorff, and R. Y. Stainer. 1968. A study of the *Moraxella* group. II. Oxidative-negative species (genus *Acinetobacter*). J. Bacteriol. **95**:1520-1541.
2. Beck, C. F., J. L. Ingraham, J. Neuhard, and E. Thomassen. 1972. Metabolism of pyrimidines and pyrimidine nucleosides by *Salmonella typhimurum*. J. Bacteriol. **110**:219-228.
3. Bendich, A. 1957. Methods for characterization of nucleic acids by base composition, p. 715-723. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 3. Academic Press Inc., New York.
4. Burton, K. 1956. A study of the conditions and mechanism of the diphenylamin reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. **62**:315-323.
5. Cohen-Bazire, G., W. R. Sistron, and R. Y. Stanier. 1957. Kinetic studies of pigment synthesis by nonsulfur bacteria. J. Cell Comp. Physiol. **49**:25-68.
6. Dale, B. A., and G. R. Greenberg. 1972. Effect of the folic acid analogue, trimetoprim, on growth, macromolecular synthesis, and incorporation of exogenous thymine in *Escherichia coli*. J. Bacteriol. **110**:905-916.
7. Davis, B. D. 1950. Studies on nutritionally deficient bacterial mutants isolated by means of pennicillin. Experientia **6**:41-50.
8. DeLey, J., H. Cattoir, and A. Reynaerts. 1970. The quantitative measurement of DNA hybridization from renaturation rates. Eur. J. Biochem. **12**:133-142.

9. Juni, E. 1972. Interspecies transformation of *A-inetobacter:* genetic evidence for a ubiquitous genus. J. Bacteriol. **112**:917-931.
10. Jyssum, S. 1971. Utilization of thymine, thymidine and TMP by *Neisseria meningitides*. 2. Lack of enzymes for specific incorporation of exogenous thymine, thymidine and TMP into DNA. Acta Pathol. Microbiol. Scand. Section B. **79**:778-788.
11. Jyssum, S., and K. Jyssum. 1970. Utilization of thymine, thymidine and TMP by *Neisseria meningitides*. 1. Growth response and uptake of labeled material. Acta Pathol. Microbiol. Scand. Section B. **78**:683-691.
12. Lederberg, J., and E. M. Lederberg. 1952. Replica plating and indirect selection of bacterial mutants. J. Bacteriol. **63**:399-406.
13. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
14. Maaløe, O., and N. O. Kjeldgaard. 1966. Control of macromolecular synthesis. Benjamin, New York.
15. Mandel, M., C. L. Schildkraut, and J. Marmur. 1968. Use of CsCl density gradient analysis for determining the guanine plus cytosine content of DNA, p. 184-195. *In* L. Grossman and K. Moldave (ed.), Methods in enzymology, vol. 12, part B. Academic Press Inc., New York.
16. Marmur, J., and P. Doty. 1962. Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. J. Mol. Biol. **5**:109-118.
17. Marshak, A., and H. J. Vogel. 1951. Microdetermination of purines and pyrimidines in biological materials. J. Biol. Chem. **189**:597-605.
18. Munch-Petersen, A. 1967. Thymidine breakdown and thymine uptake in different mutants of *Escherichia coli*. Biochim. Biophys. Acta **142**:228-237.
19. Okazaki, R., and A. Kornberg. 1964. Deoxythymidine kinase of *Escherichia coli*. I. Purification and some properties of the enzyme. J. Biol. Chem. **239**:269-274.
20. Pinter, M. 1967. Computer analysis of *Acinetobacter lwoffi* (*Moraxella lwoffi*) and *Acinetobacter anitratus* (*Moraxella glucidolytica*) strains. J. Gen. Microbiol. **46**:267-272.
21. Smith, J. T. 1967. Production of thymineless mutants in gram-negative bacteria (*Aerobacter*, *Proteus*). J. Gen. Microbiol. **47**:131-137.
22. Thornley, M. J. 1967. A taxonomic study of *Acinetobacter* and related genera. J. Gen. Microbiol. **49**:211-257.
23. Twarog, R. 1972. Enzymes of the isoleucine-valine pathway in *Acinetobacter*. J. Bacteriol. **111**:37-46.
24. Twarog, R., and G. L. Liggins. 1970. Enzymes of the tryptophan pathway in *Acinetobacter calcoaceticus*. J. Bacteriol. **104**:254-263.