Metabolism of Pyrimidine Bases and Nucleosides in the Coryneform Bacteria Brevibacterium ammoniagenes and Micrococcus luteus

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The metabolism of exogenous pyrimidine bases and nucleosides was investigated in Brevibacterium ammoniagenes and Micrococcus luteus with fluorinated analogs and radioactive precursors. Salvage of thymine and thymidine was found in M. luteus, but not in B. ammoniagenes. Exogenous uracil or uracil nucleosides, but not cytosine or cytidine nucleosides, were nucleic acid precursors for both bacteria. By examining the possible nucleoside-metabolizing enzymes, it can be suggested that the pyrimidine salvage pathways in the coryneform bacteria are different from those of members of the family Enterobacteriaceae.

Specific labeling of DNA with [2-14C]thymidine or [2-14C]thymine is not possible with Brevibacterium ammoniagenes, whereas it is possible to label the DNA of Micrococcus luteus (1). As part of our continuing study with manganese-controlled growth and DNA synthesis in the coryneform bacteria (2), we became cognizant of the need to understand thymidine metabolism in these bacteria. In this report we compare and contrast the metabolism of exogenously supplied pyrimidine bases and nucleosides in thymidine kinase (TK)-deficient (B. ammoniagenes) and TK-proficient (M. luteus) strains. The sensitivity of both strains to various 5-fluoropyrimidine analogs is also cataloged. Our data are of value to researchers for labeling DNA in organisms other than the enteric bacteria and for positive selection of mutants (e.g., fluoropyrimidine resistance) in more diverse microorganisms.

To survey the pyrimidine salvage reactions present in both strains, their sensitivity to 5-fluorouracil (FU), 5-fluorodeoxyuridine (FdUR), 5-fluorocytosine (FC), and 5-fluorocytidine (FCR) was tested. The following minimal inhibitory concentrations were determined in minimal medium in serial dilution tests (1): <1 μM FU, <5 μM FdUR, 0.5 mM FC, and 0.5 mM FCR for B. ammoniagenes and <3 μM FU, <1 μM FdUR, 0.25 mM FC, and 1 mM FCR for M. luteus. These results were confirmed by growth curve studies with a biophotometer (BIO-LOG II; Bonet-Maury, Jobin Yvon, France). The high toxicity of FdUR toward M. luteus indicates that this strain can phosphorylate the nucleoside analog to 5-fluorodeoxyuridine monophosphate which is known to be a competitive inhibitor of the thymidylate synthetase (6). Probably, the TK which is present in M. luteus (1) catalyzes this conversion.

Another way to decipher thymidine (dT)R metabolism is to ascertain which natural compounds reverse the inhibition effects of the analog FdUR. The toxicity of 5 μM FdUR toward M. luteus was abolished partially by a 20-fold molar excess of thymine (T) or dT, slightly by deoxouridine (dUR), but not at all by uridine (UR) or uracil (U). These results suggest that M. luteus can be starved for T by addition of FdUR. The extreme sensitivity of B. ammoniagenes to FU indicates a very active conversion of this pyrimidine analog to 5-fluorouracil triphosphate and incorporation into RNA, which renders the RNA nonfunctional (9, 11). The toxicity of 5 μM FU toward B. ammoniagenes was abolished completely by a 20-fold molar excess of U or UR, but was only partially overcome by an excess of dUR, and not at all by cytosine or cytidine. The high resistance toward FC and FCR observed in both strains indicates that neither transport nor metabolism of the natural pyrimidines occurs. Absence of cytosine deaminase and cytidine deaminase in both strains was confirmed by enzymatic measurements by the method of Beck et al. (4).

The salvage abilities of B. ammoniagenes were examined also by testing the requirement for pyrimidines of four mutants isolated with a block in the pyrimidine de novo synthesis (for details, see G. Auling, Habilitationsschrift, Universität Hannover, Federal Republic of Germany, 1980). These Pyr− strains grew well on minimal agar medium supplemented with U or UR. In liquid culture, their growth response to U (20 μg/ml) or UR (40 μg/ml) was identical. As growth with dUR was dubious on minimal agar, the Pyr− strain M42 was examined for growth with this nucleoside in liquid culture as well. The slow growth observed with this supplement indicates that B. ammoniagenes prefers UR to dUR as exogenous substrate for its pyrimidine requirement. For the growth of the Pyr− strains on U, UR, or dUR, we propose the following scheme, which is also based on the UR cleavage experiment (Fig. 1B) and on our enzyme assays:

UR (dUR) (outside cell) →
U (outside cell) → U (inside cell) → UMP

The Pyr− strains failed to grow with T, dTR, cytosine, cytidine, or deoxycytidine. This failure indicates that B. ammoniagenes is not able to use T and cytosine or the corresponding nucleosides as nucleic acid precursors.

Total nucleic acids were readily labeled in B. ammoniagenes and M. luteus by the use of [2-14C]U or [2-14C]UR (Fig. 1A). Under the conditions used (1, 2), a constant proportion of radioactivity was incorporated into the DNA fractions of both strains, yielding an incorporation ratio between DNA and RNA of 1:6 (B. ammoniagenes) and 1:5 (M. luteus) after 1 h of labeling. Because UR is first converted to U, as directly shown with B. ammoniagenes (Fig. 1B), it makes no difference whether U or UR is used as nucleic acid precursor for this strain. By labeling B. ammoniagenes with [8-14C]adenine, an incorporation ratio of 1:6 has previously been found (1). Therefore, we recommend either U or adenine as radioactive precursor to measure DNA synthesis in the TK-deficient strain B. ammoniagenes.

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In the TK-proficient strain *M. luteus*, specific labeling of DNA is possible. Previously, we have shown that the low efficiency of dTR incorporation can be increased by adding a 100-fold excess of deoxyguanosine. However, enhancement of dTR incorporation by addition of UR was not observed with *M. luteus*.

The incorporation of pyrimidines into nucleic acids was also tested with FU-resistant mutants of *B. ammoniagenes* selected by resistance against 1 mM FU on minimal agar. The clones M1 and M4, which incorporated less than 2% of the radioactivity from [2-14C]U or [2-14C]UR in their nucleic acids, were further examined. Preliminary transport assays (unpublished data) were performed with growing cells by the method of Jund et al. (12). When $1.7 \times 10^{-7}$ M U was offered, the wild type accumulated it intracellularly about 600-fold within 1 min. However, the FU-resistant clones M1 and M4 accumulated U only 10- to 20-fold under these conditions; they seemed to have lost their uracil permease. When $3.9 \times 10^{-7}$ M UR was offered to the clones, a similar low intracellular accumulation was found. The observed inability of the FU-resistant clones (M1 and M4) to take up UR can be explained by a very rapid enzymatic degradation of the nucleoside, which leaves no intact UR for incorporation.

To prove this hypothesis, we studied the fate of exogenous UR in culture filtrates of these clones and the wild type. Chromatography on polyethyleneimine cellulose thin-layer plates (Macherey-Nagel & Co., Düren, Federal Republic of Germany) revealed a nearly identical rapid cleavage of UR by the three strains. However, the utilization of U derived from this cleavage was different for the parent strain and the FU-resistant mutants, as the latter did not consume the free base (Fig. 1B).

The findings of the pyrimidine salvage metabolism in *B. ammoniagenes* and *M. luteus* obtained by the in vivo experiments was confirmed by enzyme measurements. Crude extracts were prepared as described earlier (1). Protein was determined by the method of Beisenherz et al. (5). Nucleoside phosphorylases were assayed in 0.2 M Tris-hydrochloride buffer (pH 7.4) by arsenolysis of pyrimidine nucleosides by the procedures of Hoffee and Blank (10) and Fribe and Holdorf (7). No phosphorolytic cleavage of dTR or dFdUR was found in the *B. ammoniagenes* wild type and could also not be detected in the Pyr" strain M42 and the FU-resistant mutant M1. These results suggest the absence of thymidine phosphorylase in *B. ammoniagenes*, which possesses no TK as already shown (1). The activity for the phosphorolytic cleavage of dUR, which is regarded as a good substrate for the enteric thymidine phosphorylases (3, 4), was very low in the *B. ammoniagenes* wild type (7 nmol min$^{-1}$ mg$^{-1}$) and undetectable in both mutants tested. However, when *B. ammoniagenes* extracts were assayed for a nucleoside hydrolase in 0.1 M 1,4-piperazine diethanesulfonic acid (pH 7.0) (Sigma Chemical Co., St. Louis, Mo.) by the method of Koszalka and Krenitsky (13), cleavage of both dUR and UR was found. Preliminary enzyme measurements after ammonium sulfate fractionation suggest that there may be two different pyrimidine nucleoside hydrolases in *B. ammoniagenes* (H. Plattner, personal communication). Apart from the hydrolytic cleavage of UR, this nucleoside is

clones M1 and M4. (B) Fate of exogenous UR during the UR labeling experiments described in (A) as analyzed by polyethyleneimine chromatography. Symbols: ■, U detected in culture filtrates of the wild type; □, U detected in culture filtrates of the FU-resistant clone M1; ●, U detected in culture filtrates of the wild type; ○, U detected in culture filtrates of the FU-resistant clone M1.
strain M42 derived from *B. ammoniagenes*. Whether this mutant was grown on U, UR, or dUR, a similar level of activity for the phosphorolytic cleavage of UR was observed (251, 300, or 290 nmol min⁻¹ mg⁻¹, respectively).

When both coryneform bacteria were assayed for the presence of a uridine kinase with radioactive substrate by the method of Beck et al. (4), activity was measurable in *B. ammoniagenes* (0.14 nmol min⁻¹ mg⁻¹) but was not detected in *M. luteus*. Despite these findings, the route functionally operative in vivo for the entry of UR into the nucleotide pools seems to be identical in both strains: cleavage of the nucleoside, yielding U, which subsequently should be converted intracellularly to UMP by an uracil phosphoribosyltransferase (Fig. 2).

Further purification of the unique pyrimidine nucleoside hydrolases of *B. ammoniagenes* is intended to clarify their specificity. As enzymatic measurements of the manganese-dependent DNA precursor formation, e.g., ribonucleotide reduction (14), in this strain were often disturbed by hydrolytic cleavage (H. Follmann, personal communication), investigations of the corresponding pyrimidine nucleoside hydrolases must be included as well.

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**LITERATURE CITED**


FIG. 2. Comparison of the presumed metabolism of exogenous thymine (T) dTR, U, UR, dUR, cytosine (C), and cytidine (CR) in *B. ammoniagenes* ATCC 6872 (A) and *M. luteus* ATCC 15932 (B). Broken arrows indicate more than one enzymatic reaction. Numbers represent salvage enzymes whose presence is established either by indirect evidence or by enzymatic measurements: 1. TK; 2. uridine kinase; 3. thymidine phosphorylase (TP); 4. UR (dUR) hydrolase; 5. uridine phosphorylase; and 6. uracil phosphoribosyltransferase. Abbreviations: CDD, cytidine deaminase; COD, cytosine deaminase; R, ribose; dR, deoxyribose; R-1-P, ribose-1′-phosphate; dR-1-P, deoxyribose-1′-phosphate.

also attacked by a uridine phosphorylase in *B. ammoniagenes*. The FU-resistant mutant which was shown to degrade UR extracellularly at the same rate as the wild type (Fig. 1B) has a high uridine phosphorylase activity (402 nmol min⁻¹ mg⁻¹) like the parent strain (406 nmol min⁻¹ mg⁻¹). Despite this rapid cleavage, *B. ammoniagenes* does not use UR as sole source of carbon due to its inability to metabolize the sugar moiety of the nucleoside.

The thymidine-incorporating reference strain *M. luteus* was shown to have a different metabolism of pyrimidine nucleosides. In addition to the TK detected earlier (1), a thymidine phosphorylase was also found. The uridine phosphorylase of *M. luteus* had a relatively weak activity, reaching only 5% that of the corresponding *B. ammoniagenes* enzyme.

Concerning induction of the pyrimidine nucleoside phosphorylases, which is well documented for members of the family *Enterobacteriaceae* (8), such a mechanism is absent in *B. ammoniagenes* and *M. luteus*, as judged from the constant enzyme activities measured under different culture conditions. When both strains were grown in the enriched minimal medium used in the uptake studies (1) either unsupplemented or supplemented with dTR, dUR, or UR, the uridine phosphorylase activity of *B. ammoniagenes* ranged from 370 to 446 nmol min⁻¹ mg⁻¹, and that of *M. luteus* ranged from 19 to 34 nmol min⁻¹ mg⁻¹. Under the same conditions, the thymidine phosphorylase activity of *M. luteus* ranged from 131 to 178 nmol min⁻¹ mg⁻¹ with dTR as substrate. Further evidence for the absence of any induction mechanism resulted from enzyme measurements of the Pyr-