Deoxynucleoside Kinases Encoded by the yaaG and yaaF Genes of Bacillus subtilis

SUBSTRATE SPECIFICITY AND KINETIC ANALYSIS OF DEOXYGUANOSINE KINASE WITH UTP AS THE PREFERRED PHOSPHATE DONOR

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The overlapping yaaG and yaaF genes from Bacillus subtilis were cloned and overexpressed in Escherichia coli. Purification of the gene products showed that yaaG encoded a homodimeric deoxyguanosine kinase (dGK) and that yaaF encoded a homodimeric deoxynucleoside kinase capable of phosphorylating both deoxyadenosine and deoxythymidine. The latter was identical to a previously characterized dAdo/dCyd kinase (Møllgaard, H. (1980) J. Biol. Chem. 255, 8216–8220). The purified recombiant dGK was highly specific toward 6-oxopurine 2'-deoxyribonucleosides as phosphate acceptors showing only marginal activities with Guo, dAdo, and 2',3'-dideoxyguanosine. UTP was the preferred phosphate donor with a $K_m$ value of 6 $\mu$M compared with 36 $\mu$M for ATP. In addition, the $K_m$ for dGuo was 0.6 $\mu$M with UTP but 6.5 $\mu$M with ATP as phosphate donor. The combination of these two effects makes UTP over 50 times more efficient than ATP. Initial velocity and product inhibition studies indicated that the reaction with dGuo and UTP as substrates followed an Ordered Bi Bi reaction mechanism with UTP as the leading substrate and UDP the last product to leave. dGTP was a potent competitive inhibitor with respect to UTP. Above 30 $\mu$M of dGuo, substrate inhibition was observed, but only with UTP as phosphate donor.

 biosynthesis of 2'-deoxyribosyl groups occurs solely through reduction of the 2'-hydroxyl group of ribonucleoside di- or triphosphates, catalyzed by ribonucleotide reductases (1, 2). In addition, a number of organisms possess deoxynucleoside kinases that provide a salvage pathway for the utilization of preformed deoxynucleosides as precursors of DNA. Because the cytotoxicity of a large variety of deoxynucleoside analogs depends on the conversion of these compounds to the corresponding deoxynucleotide analogs, characterization of the substrate specificity and regulation of deoxynucleoside kinases from various sources have received considerable attention (3).

Thymidine kinase (dTK),1 which can generally use both Thd and dUrd as substrates, is widely distributed in both prokaryotes and eukaryotes. In contrast, only relatively few genera have been shown to express deoxyguanosine kinase (dGK), dCTP, and deoxyadenosine kinase (dAK) activities. From mammalian tissues four deoxynucleoside kinases, TK1, TK2, dCK, and dGK, with overlapping substrate specificities, have been characterized (for review see Ref. 3). TK1 and TK2 are pyrimidine-specific, phosphorylating Thd and dUrd. TK2 can in addition use dCyd as a substrate (4–6). dCK phosphorylates dCyd, dAdo, and dGuo (7, 8), and the mitochondrial dGK is specific for dGuo and dAdo (9, 10). A deoxynucleoside kinase with a very different substrate specificity was recently characterized from Drosophila melanogaster. In this organism a single homodimeric enzyme (Dm-dNK) is capable of phosphorylating all four deoxynucleosides, although with widely different efficiencies (11–13).

Among eubacteria only two genera, Lactobacilli (14) and Bacilli (15), have been shown to phosphorylate all four deoxynucleosides, whereas it has been established that a number of bacteria, including Escherichia coli and Salmonella enterica serovar Typhi, are lacking dGK, dAK, and dCK activities (16). In Lactobacillus acidophilus strain R26, a strain that appears to lack a functional ribonucleotide reductase (14), the deoxynucleoside kinase activities are organized as three enzymes. In addition to a separate dTK, the remaining three activities are located on two heterodimeric proteins, dGK/dAK and dCK/dGK, with each subunit being highly specific for the individual substrates, and specifically feedback inhibited by its respective dNTP end-product (17). The three subunits of the two heterodimeric enzymes are encoded by two tandem genes, dak and dgk, where dak encodes the dAK subunit of both enzymes, and dgk encodes both dGK and dCK. The only difference between the amino acid sequences of the two latter subunits is that dCK lacks the N-terminal amino acid residues 2 and 3. The mechanism responsible for the co- or post-translational deletion event has not yet been identified (18). In contrast, the organization of the activities in Bacillus subtilis is quite different. dTK and dGK are genetically distinct and different from the enzyme that phosphorylates dAdo and dCyd (15).

The present report concerns the molecular cloning of two overlapping B. subtilis genes encoding two homodimeric enzymes, dGK and dAK, and describes the purification and characterization of the recombinant dGK overexpressed in E. coli. The dAK/dCK enzyme appeared to be identical to the deoxyadenosine/dectydide kinase previously purified and characterized from B. subtilis (15).

EXPERIMENTAL PROCEDURES

Materials

Tris (TRIZMA base), BSA, nucleotides, and nucleosides were obtained from Sigma-Aldrich, Denmark, and 2',3'-dideoxynucleosides were gifts from Dr. H. G. Ihlenfeldt, Roche Molecular Biochemicals.

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‡ The abbreviations used are: dTK, thymidine kinase; BSA, bovine serum albumin; Bs-dGK, Bacillus subtilis deoxyguanosine kinase; dAK, deoxyadenosine kinase; dCK, deoxycytidine kinase; dGK, deoxyguanosine kinase; Dm-dNK, Drosophila melanogaster deoxynucleoside kinase; PAGE, polyacrylamide gel-electrophoresis; PCR, polymerase chain reaction; bp, base pair(s).
Phosphoenolpyruvate, pyruvate kinase, and lactate dehydrogenase were from Roche Molecular Biochemicals. Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs, and [γ-32P]ATP deoxyadenosine was from Moravek Biochemicals. Dyematrix Blue A and Red A were from Amicon, and Sephacryl S-300 was from American Pharmacia-Biotech. DE-81 ion exchange paper was from Whatman Ltd., United Kingdom. SDS-PAGE low range molecular weight standards were purchased from Bio-Rad Laboratories.

Methods

Bacterial Strains and Growth Conditions—E. coli SØ5110 (MC1061 cdd::Tn10) (19) was used as host strain and pUC18 as cloning vector throughout. Cells were grown at 37 °C in LB medium (20). When required, ampicillin was added to the medium at 100 μg/ml.

DNA Techniques—The methods used for preparing chromosomal DNA and for transforming E. coli have been described previously by Sambrook et al. (21). Plasmids were isolated from E. coli by the alkaline/SDS lysis procedure (22). Fragments from digested plasmid DNA were isolated and purified from agarose gels by the QIAquick gel extraction kit (Qiagen, Germany). Endonuclease digestion and ligation of DNA was done according to the recommendations of the suppliers. DNA was sequenced by the chain termination method (23) on polymerase chain reaction (PCR) products, using the BigDye Terminator cycle sequencing kit (PE Applied Biosystems, Warrington, United Kingdom) and the ABI Prism 310 genetic analyzer (PE Applied Biosystems). DNA gel band sizes were determined by comparison with a ladder of DNA markers determined by gel electrophoresis as described (24) using BSA as a standard.

Data Treatment—Km and Vmax values were obtained by fitting steady-state kinetic data to the rate equation for a sequential Bi Bi mechanism with the BioSoft program UltraFit 3.0 for Macintosh. K values were calculated from secondary plots of inhibitor concentration versus intercept or slope of the double-reciprocal plots using linear least-squares regression analysis to determine the best-fit line describing the data. Mass Spectrometry—The mass spectrometry was performed by Dr. Jette Wagtberg Sen at the Statens Seruminstitut, Copenhagen, using a PerSeptive Biosystems Ultraflextreme time-of-flight spectrometer (PerSeptive Biosystems).

Denaturing Polyacrylamide Gel Electrophoresis—Protein samples were incubated for 5 min at 100 °C in 50 mM Tris- HCl, pH 8.8, 10% glycerol, 2% SDS, 0.1% bromphenol blue and applied on a 15% polyacrylamide-SDS gel (25). Gels were run at 40 mA for 70 min and silver-stained (26).

RESULTS

Cloning of the B. subtilis yaaG and yaaF Genes—The amino acid sequences of dAK and dGK from L. acidophilus, deduced from the nucleotide sequence of the dak/dgk tandem genes (27), were used to search the B. subtilis genome sequence (28) for open reading frames encoding putative deoxynucleoside kinases. Two genes, yaaG and yaaF, were identified at coordinates 22497–23576 bp, which encoded putative polypeptides with 25–32% amino acid sequence identity to the L. acidophilus deoxynucleoside kinases. The nucleotide sequence indicated that the TGA stop codon of yaaG overlapped 2 bp with the ATG of the start codon of yaaF, and that both open reading frames were preceded by typical B. subtilis Shine-Dalgarno sequences (29). The deduced amino acid sequences of the two gene products were 30% identical.

The putative yaaG/yaaF operon was amplified from B. subtilis genomic DNA by PCR and cloned into pUC18, resulting in pRA8 (Fig. 1). Subsequently, yaaG and yaaF were subcloned separately in pUC18 using the internal PacI and EcoRI sites of pRA8, yielding plasmids pRA9 and pRA5, respectively (Fig. 1). In all constructs the cloned genes were transcribed from the lac
promoter (lacP) of the vector and translated from the native ribosomal binding regions. DNA sequencing of the cloned fragments of pRA9 and pRA5 confirmed the published genomic sequence and showed that they encoded potential polypeptides of 207 and 217 amino acid residues, respectively. SDS-PAGE of total proteins from cultures of E. coli harboring pRA9 or pRA5 revealed a major band with a mobility corresponding to a 24-kDa polypeptide, in accordance with the molecular masses of the yaaG and yaaF gene products as deduced from the nucleotide sequence (data not shown).

Identification of the yaaG and yaaF Gene Products—Crude cellular extracts of E. coli SØ5110 harboring pRA8, pRA9, and pRA5 were assayed for deoxynucleoside kinase activity with dGuo, dAdo, and dCyd as substrates (Table I). The results suggested that yaaG encoded a kinase specific for deoxyguanosine, whereas the yaaF gene product was capable of phosphorylating both deoxyadenosine and deoxyctydine.

To establish whether all three kinase activities were part of a single heterooligomeric enzyme in conditions where both yaaG and yaaF were present in the cells, crude extracts of E. coli SØ5110/pRA8 were subjected to both ammonium sulfate fractionation and dye-affinity chromatography. Table II shows that the kinase activities for dGuo, dAdo, and dCyd copurified upon ammonium sulfate precipitation and on RedA dye chromatography. However, on a BlueA dye column deoxyguanosine kinase activity eluted clearly before the deoxyadenosine and deoxyctydine kinase activities. Together with the results presented in Table I, this indicated that the gene products of yaaG and yaaF formed two physically distinct enzymes, dGK and dAK/dCK. Preliminary characterization of the recombinant dAK/dCK from the BlueA dye column indicated that it was identical to the deoxyadenosine/deoxyctydine kinase previously purified and characterized from B. subtilis (15).

Purification of Recombinant dGK—Having established that deoxyguanosine kinase was encoded by the yaaG gene, the recombinant enzyme was purified from cells of E. coli SØ5110 harboring pRA9. Because preliminary experiments showed that ammonium sulfate precipitation resulted in heavy losses, this step was avoided. The final procedure adopted involved fractionation by BlueA dye affinity chromatography and separation according to hydrophobicity on a phenyl-Sepharose column producing an enzyme that was >99% pure as judged by SDS-PAGE, with an overall yield of 25% (Table III).

Molecular Weight—The molecular mass of the subunit was determined to be 24,147 Da by electrospray mass spectrometry (data not shown), in accordance with the molecular mass of the yaaG gene product deduced from the DNA sequence (24,145 Da). The molecular mass of purified recombinant dGK was about 49 kDa as estimated by gel filtration on a Sephacryl
more efficient than the other NTPs. With dGTP no measurable activity was observed (data not shown).

Steady-state Kinetics—Initial velocity experiments were carried out at varying concentrations of the four individual ribonucleoside triphosphates and fixed concentrations of dGuo. For each of the NTPs, double reciprocal plots of the initial rate as a function of the NTP concentration for various fixed dGuo concentrations yielded a series of intersecting lines, diagnostic of a sequential reaction mechanism (data not shown). The kinetic constants obtained (Table V) were fitted to the rate equation for a sequential Bi Bi reaction mechanism (data not shown). The data obtained were fitted to the rate equation for a sequential Bi Bi mechanism. The kinetic constants obtained (Table V) showed that the true $K_m$ values for the various NTPs were quite similar, whereas large variations in the true $K_m$ values for the four NTPs were observed. The $k_{cat}/K_m$ measure of substrate efficiency indicated a preference for UTP as phosphate donor by a factor of 4–6. Furthermore, the data indicated that the $K_m$ for dGuo was highly dependent on the nature of the phosphate donor (Table V). In the presence of UTP the true $K_m$ for dGuo was 0.6 $\mu$M compared with 6.5 with ATP. This synergism between the high affinity for UTP and the low $K_m$ for UTP with UTP as phosphate donor makes UTP 50–60 times more efficient than ATP as a phosphate donor. In all further kinetic studies UTP was employed as phosphate donor.

Product inhibition studies with dGuo and UTP as substrates were performed to determine the binding order of substrates and products (Fig. 2, A–D; Table VI). The secondary plots of the slopes or intercepts versus the concentration of inhibitor were in all cases linear (see insets in Fig. 2, A–D). With UTP as the variable substrate, inhibition by UDP was competitive (Fig. 2A), whereas inhibition by dGMP was noncompetitive with $K_i$, being of the same order of magnitude as $K_m$ for UTP, and $K_i$ about 25-fold higher (Fig. 2B). With dGuo as the variable substrate, both products produced noncompetitive inhibition (Fig. 2, C and D). This inhibition pattern suggested that dGK followed a sequential ordered reaction mechanism in which UTP had to bind before dGuo, and dGMP was leaving the enzyme complex before UDP. Such an ordered reaction mechanism predicted uncompetitive inhibition by dGMP with UTP as variable substrate, under conditions of full saturation with respect to the second substrate, dGuo. Because of substrate inhibition exerted by dGuo (see below) this prediction could not be tested. Whether the reaction with other NTPs as phosphate donors followed the same ordered reaction mechanism was not investigated.

Substrate inhibition was observed by dGuo at concentrations above 30 $\mu$M. As shown in Fig. 3, the inhibition was exclusively observed with UTP as phosphate donor, and it was found to be linear (Fig. 3, inset). This indicated that the inhibition resulted from the formation of a dead-end complex of dGuo with a form of the enzyme that it is not supposed to react with (30). The inability of high UTP concentrations to reverse the inhibition (data not shown) suggested that dGK, the second substrate in the ordered reaction, combined with the E-UDP complex to form the dead-end complex.

Inhibition of dGK by dGTP—As mentioned above, dGTP was the only naturally occurring NTP that did not function as phosphate donor for dGK. In contrast, dGTP was a potent competitive inhibitor of the enzyme with respect to UTP, with a $K_i$ value of 0.4 $\mu$M (data not shown). The pattern of dGTP inhibition with dGK being the variable substrate was noncompetitive at lower dGuo concentrations with $K_i$ and $K_m$ values of 1.6 and 0.7 $\mu$M, respectively.

### DISCUSSION

The genetic and enzymatic basis for the ability of _B. subtilis_ to phosphorylate dCyd, dAdo, and dGuo was established by cloning and overexpressing the overlapping _yaaG_ and _yaaF_ genes in _E. coli_, an organism unable to phosphorylate deoxyribonucleosides other than dThd (16). Simultaneous expression of both genes yielded two separable enzymes, one specific for dGuo and the other capable of phosphorylating both dCyd and dAdo. Expression of each gene separately showed that _yaaG_ encoded dGK and _yaaF_ encoded dCK/dAK, and that both enzymes were homodimeric proteins. Preliminary studies indicated that dCK/dAK was identical to the previously characterized deoxycytidin kinase of _B. subtilis_ (15). Based on these findings we propose that the _yaaG_ and _yaaF_ genes be renamed as _dgk_ and _dak_, respectively.

The recombinant _B. subtilis_ dGK was purified to homogeneity and characterized. Compared with other enzymes with deoxyguanosine kinase activity the selectivity of _Bs-dGK_ for the nucleoside substrate is unique. Only 6-oxopurine 2'-deoxyribonucleosides (dGuan and dIno) are phosphorylated by the enzyme at significant rates, with a $K_m$ for dGuo of 0.6 $\mu$M and a $k_{cat}$ of 1.4 s$^{-1}$. The homodimeric mammalian mitochondrial dGK, which in addition to dGuo and dIno can use dAdo as a substrate, shows $K_m$ and $k_{cat}$ values of 7.6 $\mu$M and 0.002 s$^{-1}$, respectively, with dGuo as a substrate (9), and the homodimeric human dCK, which uses dCyd, dAdo, and dGuo, phosphorylates dGuo with a $K_m$ of 150–430 $\mu$M and a $k_{cat}$ of...
0.4–6.0 s\(^{-1}\) (7, 31). Recently, a monomeric multisubstrate dNK from Drosophila melanogaster (Dm-dNK) was characterized (12). Its \(K_m\) for dGuo was found to be very high (654 \(\mu\)M), but at the same time the enzyme displayed the highest \(k_{cat}\) reported for any deoxynucleoside kinase (18 s\(^{-1}\)). The specificity constant \(k_{cat}/K_m\) for dGuo phosphorylation by Bs-dGK is thus \(10^2\) to \(10^4\) higher than the corresponding values of the various eukaryotic deoxyguanosine kinases. Expression in E. coli of the L. acidophilus dgk gene, encoding the dGK subunit of the heterodimeric dAK/dGK, results in the production of a loosely associated homodimeric dGK, with significant similarity to Bs-dGK regarding its specificity toward the nucleoside sub-

### TABLE V

**Kinetic constants for Bs-dGK with different NTPs as phosphate donors**

| NTP  | \(V_{max}\) \(a\) | \(k_{cat}\) \(b\) | \(K_m\) \(a\) | \(k_{cat}/K_m\) \(c\) |
|------|-------------------|-----------------|-----------|-----------------|
| UTP  | 3.5 s\(^{-1}\)    | 1.41 \(\mu\)M  | 2.4 \(\times\) 10\(^{5}\) (1.00) | 6.5 \(\mu\)M  |
| CTP  | 5.6 s\(^{-1}\)    | 2.25 \(\mu\)M  | 6.4 \(\times\) 10\(^{4}\) (0.27) | 1.7 s\(^{-1}\) |
| ATP  | 3.8 s\(^{-1}\)    | 1.53 \(\mu\)M  | 4.3 \(\times\) 10\(^{4}\) (0.18) | 6.5 \(\mu\)M  |
| GTP  | 4.2 s\(^{-1}\)    | 1.69 \(\mu\)M  | 3.7 \(\times\) 10\(^{4}\) (0.15) | 10.4 \(\mu\)M  |

\(a\) \(V_{max}\) and \(K_m\) values were obtained from double-reciprocal plots of initial velocity experiments as described under "Methods." For each NTP, five different concentrations of dGuo and five different concentrations of the triphosphate were used.

\(b\) The turnover number, \(k_{cat}\), was calculated per subunit.

\(c\) Numbers in parenthesis are relative values.

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**FIG. 2. Product inhibition studies.** Double-reciprocal plots of product inhibition experiments. Insets are replots of slopes and intercepts against inhibitor concentrations. A, inhibition by UDP at various UTP concentrations; B, inhibition by dGMP at various UTP concentrations; C, inhibition by UDP at various dGuo concentrations; D, inhibition by dGMP at various dGuo concentrations. Inhibitor concentrations in micromolar are shown at the end of each line. \(1/v\) is (units/mg)\(^{-1}\).

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strate. However, the specificity constant of this "unnatural" dGK is only 1/20th of that of Bs-dGK (18).

In contrast to its strict phosphate acceptor requirement, Bs-dGK can use most NTPs as phosphate donors. However, the results presented in Table V indicate that UTP is the preferred phosphate donor. Compared with the other NTPs the efficiency of UTP results from a 6-fold lower $K_m$ for UTP than for the other NTPs and a much lower $K_m$ for dGuo when UTP is employed as donor. The steady-state kinetic analysis of the reaction with dGuo and UTP indicated that the reaction follows an Ordered Bi Bi mechanism with UTP as the leading substrate and release of UDP last (Scheme 1). The competitive inhibition by dGTP with respect to UTP is consistent with an ordered addition of substrates with UTP binding first (32). The substrate inhibition data (Fig. 3) are also consistent with the proposed kinetic model and suggest formation of the dead-end complex E-UDP-dGuo. The observation that substrate inhibition with dGuo only occurred with UTP as phosphate donor shows that the reaction with other NTPs as phosphate donors may follow a different kinetic mechanism.

It was previously observed that UTP, CTP, and dTTP were more efficient phosphate donors than ATP for the bovine liver mitochondrial dGK at physiological pH (33), and that UTP was the preferred phosphate donor for human dCK with dCyd and dAdo as acceptors (34, 35). More recently, a detailed kinetic analysis of human dCK with dCyd and UTP was reported (36). It showed that the preference for UTP as phosphate donor was primarily due to large differences in the true $K_m$ values for the phosphate donor with values of 1.2 and 54 $\mu M$ for UTP and ATP, respectively. Only a 2-fold difference in the true $K_m$ values for dCyd was observed (0.5 and 1.0 $\mu M$ with UTP and ATP, respectively). The kinetic mechanism of dCyd phosphorylation also appeared to depend on the phosphate donor showing a random Bi Bi reaction sequence with ATP but an ordered addition-random release sequence with UTP (31, 36). Unfortunately, the kinetic properties of the enzyme with dAdo or dGuo as substrates were not investigated.

Most deoxynucleoside kinases are end-product-inhibited by the corresponding dNTP, and it has been shown in a number of cases that the dNTP inhibitor behaves like a bisubstrate analog.

### Table VI

| Product | Variable substrate | Fixed substrate | Type of inhibition | $K_i$ $\mu M$ | $K_{ii}$ $\mu M$ |
|---------|--------------------|-----------------|-------------------|-------------|-------------|
| UDP     | UTP (1–1000 $\mu M$) | dGuo (3 $\mu M$) | Competitive       | 63          |             |
| dGMP    | UTP (1–1838 $\mu M$) | dGuo (1 $\mu M$) | Noncompetitive    | 8           | 207         |
| UDP     | dGuo (0.5–8 $\mu M$) | UTP (10 $\mu M$) | Noncompetitive    | 218         | 95          |
| dGMP    | dGuo (0.5–8 $\mu M$) | UTP (10 $\mu M$) | Noncompetitive    | 60          | 140         |

*Derived from the inserted secondary plots in Fig. 2, A–D.*

![Fig. 3. Substrate inhibition by dGuo with various NTPs as phosphate donors.](http://www.jbc.org/)

**Scheme 1**

![Diagram of Scheme 1](http://www.jbc.org/)

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(17, 37, 38). The kinetics of dGTP inhibition and the observation that all NTPs and dNTPs except dGTP function as phosphate donors, suggest that dGTP inhibition of Bs-dgk is due to binding of the inhibitor to both the deoxynucleoside acceptor site and the triphosphate binding site of UTP. The intracellular amounts of nucleoside triphosphates in B. subtilis have been measured previously (39). For UTP and dGTP they were estimated to be 0.15 and 1.6 nmol/mg of dry weight, which corresponds to 188 μM and 2 mM respectively. 2 Insertion of these values, the $K_v$ for dGTP of 0.4 μM, and the $K_m$ for UTP of 6 μM, into the rate equation for competitive inhibition shows that the enzyme would be inhibited by ~60% under these conditions, indicating that this feedback control may be of physiological importance.

Recently, the nucleotide sequence of the entire Deinococcus radiodurans genome was determined (40). It revealed that this Gram-positive bacterium contains two overlapping genes encoding putative polypeptides with 32 and 35% identity to the yaaG and yaaF genes of B. subtilis, respectively. Thus, D. radiodurans may represent a third genus of eu bacteria possessing dGuo, dAdo, and dCyd kinase activities. It is noteworthy that homologous genes have so far not been identified in Gram-negative bacteria.

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2 The calculations are based on the assumption that 1 mg of bacterial dry weight corresponds to 2 × 10^8 cells and that the volume of one cell is 4 μm^3.
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