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# EFFECT OF ACETIC ACID ON POLY-(3-HYDROXYBUTYRATE-CO-3-HYDROXYVALERATE) SYNTHESIS IN RECOMBINANT *ESCHERICHIA COLI*

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**Abstract**—The synthesis of poly-(3-hydroxybutyrate-co-3-hydroxyvalerate) [P(3HB-co-3HV)] copolymer by recombinant *Escherichia coli* was studied in the medium containing glucose and valeric acid as carbon sources. A recombinant *E. coli* strain (*fadR atoC*) harboring a stable high-copy number plasmid containing the *Alcaligenes eutrophus* polyhydroxyalkanoate (PHA) biosynthesis genes was constructed for the production of the copolymer P(3HB-co-3HV). Accumulation of acetic acid not only had a detrimental effect on cell growth but also decreased the flux of acetyl-CoA into the P(3HB-co-3HV) biosynthetic pathway. Reducing specific growth rate by increasing the initial acetic acid concentration resulted in enhanced copolymer synthesis due to less accumulation of acetic acid. Initial acetic acid concentration of 50 mM was found to be optimal at 20 g/*l* glucose and 20 mM valeric acid concentration. The fraction of 3-hydroxyvalerate (3HV) increased with decreasing growth temperature. The ratios of 3HV to 3HB in the copolymer could be controlled by altering the concentrations of valeric acid and glucose in the medium. Catabolite repression was in part responsible for the inefficient copolymer synthesis. Various nutritional components were examined for their ability to relieve catabolite repression. An addition of oleic acid resulted in threefold increase of the 3HV fraction in the copolymer. An addition of a small amount of tryptone and peptone considerably promoted P(3HB-co-3HV) synthesis.

*Key words: Poly-(3-hydroxybutyrate-co-3-hydroxyvalerate), Polyhydroxyalkanoate, Escherichia coli, Acetic Acid*

## INTRODUCTION

Polyhydroxyalkanoic acid (PHA) is an intracellular energy reserve material accumulated by a variety of bacteria under certain unbalanced growth conditions [Anderson and Dawes, 1990]. Among these polyesters poly-3-hydroxybutyric acid, P(3HB), is the best known thermoplastic polymer accumulated by a wide range of organisms [Haywood et al., 1989]. The copolymer of 3-hydroxybutyric and 3-hydroxyvaleric acids, P(3HB-co-3HV), has been of particular interest because it is more flexible than the P(3HB) homopolymer with better material properties [Byrom, 1987]. Some bacterial strains have been shown to accumulate P(3HB-co-3HV) copolymer when propionic or valeric acid was supplied as a co-substrate with a main carbon source [Holmes, 1985].

The PHA biosynthesis genes of *Alcaligenes eutrophus* H16 have been cloned and studied in detail [Schubert et al., 1988; Slater et al., 1988; Peoples and Sinskey, 1989a; Peoples and Sinskey, 1989b]. Heterologous expression of a functionally active *A. eutrophus* PHA biosynthetic pathway in recombinant bacteria has been reported, and plasmids harboring the *A. eutrophus phbCAB* operon conferred the capability to synthesize and accumulate PHA to *E. coli* [Schubert et al., 1988; Slater et al., 1988].

We previously reported production of 81 g/*l* of P(3HB) in 39h by fed-batch culture of recombinant *E. coli* harboring the *A. eutrophus* PHA biosynthesis genes, resulting in the high productivity of 2.08 g P(3HB)/*l*-h [Lee et al., 1994b]. Use of recombinant *E. coli* for the production of P(3HB) has several advantages such as fast growth, a large amount of polymer accumulation, an ability to use various carbon sources, well-established high cell density culture techniques, and the lack of depolymerases [Lee et al., 1994a; Lee and Chang, 1995].

However, a copolymer containing 3-hydroxyvalerate (3HV) units was not synthesized by recombinant *E. coli* in the condition that allowed synthesis of P(3HB-co-3HV) in *A. eutrophus*. Recently, Slater et al. [Slater et al., 1992] reported synthesis of P(3HB-co-3HV) using a mutant *E. coli* LS5218 (*fadR atoC*), which can constitutively express the enzymes involved in fatty acid utilization. Their results clearly showed that the lack of P(3HB-co-3HV) synthesis was due to the inability of *E. coli* to utilize short-carbon-length fatty acids such as propionic or valeric acid. The effects of varying glucose and propionic acid concentrations as well as the time of their addition on the synthesis of the copolymer were investigated in detail [Slater et al., 1992]. With an aim to produce high levels of P(3HB-co-3HV) by fed-batch culture of recombinant *E. coli*, we investigated the effect of several metabolites on copolymer synthesis in the medium containing high concentrations of salts, which simulates the initial fed-batch condition. Valeric acid was chosen as a co-substrate since it allowed more accumulation of P(3HB-co-3HV) than propionic acid (results not shown). The biosynthetic pathway of P(3HB-co-3HV) from valeric acid was suggested as follows. Acetyl-CoA and propionyl-CoA are generated through the β-oxidation cycle from valeric acid. D(-)-3-hydroxyvaleryl-CoA is formed by a reaction of propionyl-CoA with acetyl-CoA or by a direct pathway from valeryl-CoA without decomposition, and D(-)-3-hydroxybutyryl-CoA is formed from two molecules of acetyl-CoA. P(3HB-co-3HV) is synthesized by the polymerization of D(-)-3-hydroxvaleryl-CoA and D(-)-3-hydroxybutyryl-CoA

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moieties [Doi, 1990].

In this paper the effect of initial acetic acid concentration and acetic acid accumulation on the synthesis of P(3HB-co-3HV) was examined. The effects of varying carbon source concentration and supplementing cyclic adenosine monophosphate (c-AMP), various nitrogen sources, and oleic acid on the copolymer synthesis are also reported.

## MATERIALS AND METHODS

### 1. Bacterial Strain and Plasmid

*E. coli* LS5218 (*fadR601 atoC2*) was obtained from the *E. coli* Genetic Stock Center at Yale University. A stable high copy number plasmid pSYL104 containing the *A. eutrophus* PHA biosynthesis genes has been previously described [Lee et al., 1994b].

### 2. Culture Conditions

*E. coli* LS5218 was grown in a minimal medium with glucose and valeric acid as carbon sources. The culture medium contained per liter: $(NH_4)_2SO_4$, 1 g; $KH_2PO_4$, 13.3 g; citric acid, 1.7 g; $MgSO_4 \cdot 7H_2O$, 1.2 g; trace element solution, 10 ml. The trace element solution contained per liter: $FeSO_4 \cdot 7H_2O$, 10 g; $CaCl_2 \cdot 2H_2O$, 2 g; $ZnSO_4 \cdot 7H_2O$, 2.25 g; $MnSO_4 \cdot 4\text{-}5H_2O$, 0.5 g; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.1 g; $Na_2B_4O_7 \cdot 10H_2O$, 0.02 g; $CuSO_4 \cdot 5H_2O$, 1 g; HCl, 10 ml. Ampicillin was added at a concentration of 100 μg/ml. Experiments were conducted in a 250 ml flask containing 100 ml medium at 37℃ and 250 rpm.

Both the glucose and valeric acid used as carbon sources had inhibitory effects on cell growth and P(3HB-co-3HV) formation. Glucose might inhibit the expression of enzymes necessary for the conversion of valeric acid to valeryl-CoA, thereby decreasing the 3HV fraction in the copolymer [Weeks et al., 1969]. Valeric acid at the concentration higher than 20 mM was found to be toxic to cells. Therefore, it seems to be important to initiate polymer production by the addition of both carbon sources late in exponential growth phase as suggested by Slater et al. Cells were first grown with acetic acid as the sole carbon source to induce fatty-acid-utilizing enzymes and to avoid catabolite repression [Maloy and Nunn, 1982]. Glucose and valeric acid were added when the optical density at 600 nm reached 0.8 or 1.3. Yeast extract, tryptone, peptone (Difco Laboratories, Detroit, Michigan), and casamino acids (Sigma Chemical Co., St. Louis, Missouri) were used as complex nitrogen sources as described later.

### 3. Analytical Procedures

Cell growth was temporarily monitored by measuring the $OD_{600}$ with a spectrophotometer (Beckman DU-65, USA). To measure cell mass (concentration), a 5-10 ml of culture broth was centrifuged and dried to a constant weight at 80℃.

PHA concentration was determined by gas chromatography (Varian 3300, USA) with n-butyric acid as an internal standard [Braunegg et al., 1978]. Glucose and acetic acid concentrations in the medium were measured by high performance liquid chromatography equipped with the Aminex HPX-87H column (Bio-Rad Laboratories Ltd., USA) and a refractive index detector (L-3300, Hitachi, Japan), using 0.01 N $H_2SO_4$ as the mobile phase.

## RESULTS AND DISCUSSION

### 1. Effect of Acetic Acid on the Formation of P(3HB-co-3HV)

One of the difficulties often arising in recombinant *E. coli* fermentation is the formation of unwanted by-products [Luli and Strohl, 1990]. Growth of *E. coli* in the presence of excess glucose even under aerobic conditions causes the formation of acidic by-products, of which acetic acid is the most predominant. This glucose-mediated aerobic acidogenesis, known as the bacterial Crabtree effect, is most readily observed at high growth rates in the medium containing glucose at high concentration [Meyer et al., 1984]. Accumulation of acetic acid has been known to be detrimental to cell growth and product formation in recombinant fermentation [Pan et al., 1987].

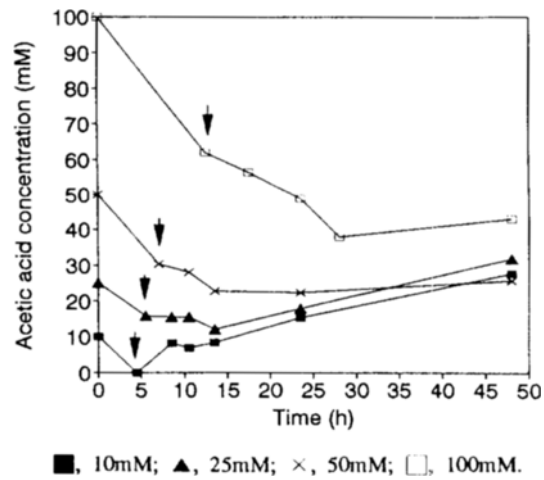
In our system acetic acid formation is one of the several competing pathways that reduce the amount of acetyl-CoA available for PHA synthesis [Lee et al., 1994a; Lee and Chang, 1995]. It was reasoned that less acetic acid would be formed by decreasing growth rate. Since cells are initially grown with acetic acid as a sole carbon source for the efficient synthesis of P(3HB-co-3HV), it was thought that growth rate could be decreased by simply increasing the initial acetic acid concentration. Furthermore, it is good to find that the strain LS5218 used in this study can utilize more exogenous acetic acid than the wild type strain due to the elevated levels of glyoxylate shunt enzymes [Maloy and Nunn, 1981].

The initial acetic acid concentration was varied from 10 to 100 mM (Table 1). The specific growth rate decreased with the increasing initial acetic acid concentration as expected. However, final cell mass and PHA concentration increased with higher initial acetic acid concentration. The 3HB concentration obtained

**Table 1. Effect of initial acetic acid concentration on cell growth and P(3HB-co-3HV) synthesis[a]**

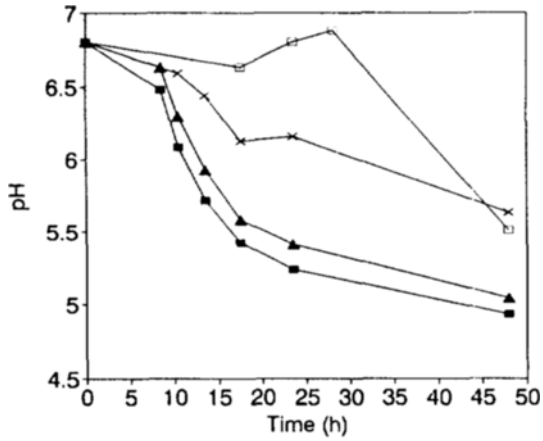
| | Initial acetic acid concentration (mM) | | | |
|---|---|---|---|---|
| | 10 | 25 | 50 | 100 |
| $\mu_{max}$ ($h^{-1}$) | 0.623 | 0.472 | 0.384 | 0.219 |
| DCW (g/l) | 1.42 | 1.76 | 2.18 | 3.78 |
| PHA (g/l) | 0.29 | 0.46 | 0.72 | 0.74 |
| PHA content (wt%) | 20.4 | 26.1 | 33.0 | 19.6 |
| 3HV fraction (mol%) | 27.6 | 19.6 | 11.1 | 8.1 |

[a]Glucose (20 g/l) and valeric acid (20 mM) were added when the culture reached an $OD_{600}$ of 0.8.




■, 10mM; ▲, 25mM; ×, 50mM; □, 100mM.

**Fig. 1. Time profile of acetic acid concentration in the flask cultures with varying initial acetic acid concentrations of: ■, 10 mM; ▲, 25 mM; ×, 50 mM; □, 100 mM. The arrows indicate the time of glucose and valeric acid addition.**




■, 10mM; ▲, 25mM; ×, 50mM; □, 100mM.

**Fig. 2. Time profile of pH in the flask cultures with varying initial acetic acid concentrations of: ■, 10 mM; ▲, 25 mM; ×, 50 mM; □, 100 mM.**

with 50 mM acetic acid was three times higher than that obtained with 10 mM acetic acid. Further increase of acetic acid to 100 mM gave higher cell mass without concomitant increase of PHA concentration, resulting in the decrease of PHA content. The 3HV concentration did not increase as the initial acetic acid concentration increased, resulting in the decrease of 3HV fraction of the copolymer.

To test our hypothesis that the increase of PHA synthesis is due to the reduction of acetic acid formation, acetic acid concentration was measured during the culture (Fig. 1). During the incipient phases acetic acid concentration decreased because it was used as the sole carbon source. Acetic acid formation was initiated when supplemented glucose started to be utilized. As the initial acetic acid concentration was increased up to 50 mM, less acetic acid was formed from glucose. With the initial acetic acid concentration of 10 mM, acetic acid concentration increased from 0 mM at 4.5 h to 25 mM at 48 h. However, there was no notable increase of acetic acid concentration when the initial acetic acid concentration was 50 mM. These results suggest that the surplus intracellular acetyl-CoA resulting from the decrease of acetic acid formation could be possibly metabolized into the PHA biosynthetic pathway. The time profiles of pH were also examined. When the initial acetic acid concentration was high, pH declined more slowly (Fig. 2). This also proved that a high level of initial acetic acid concentration resulted in less accumulation of acetic acid. The optimal level of initial acetic acid was 50 mM to produce P(3HB-co-3HV) at 20 g/*l* glucose and 20 mM valeric acid concentration in recombinant *E. coli*. The relation between the specific growth rate and acetic acid formation in chemostat culture was studied by several groups. Acetic acid was produced at high growth rates ($\mu_{max}$>0.35-0.45 $h^{-1}$) in a defined medium [Meyer et al., 1984; Reiling et al., 1985]. In our system similar results were obtained; acetic acid production increased at a growth rate higher than 0.4 $h^{-1}$.

When the cell growth was deterred by decreasing the growth temperature from 37 to 27℃ at the $OD_{600}$ of 0.8, acetic acid accumulation decreased to 18 mM at 48 h. The 3HV fraction in the copolymer increased from 11.1 to 22.1 mol% with a decrease in growth temperature. The reason for the increase in 3HV is not clear at this time, but membrane permeability might have been changed to allow better uptake of valeric acid.

**Table 2. Effect of valeric acid concentration on P(3HB-co-3HV) synthesis[a]**

| | Valeric acid concentration (mM) | | | | |
|---|---|---|---|---|---|
| | 0 | 10 | 20 | 30 | 40 |
| DCW (g/*l*) | 2.73 | 3.06 | 3.21 | 2.90 | 2.92 |
| PHA (g/*l*) | 0.93 | 1.36 | 1.56 | 1.26 | 1.25 |
| PHA content (wt %) | 34.1 | 44.4 | 48.6 | 43.4 | 42.8 |
| 3HV fraction (mol %) | nd[b] | 13.2 | 14.7 | 17.5 | 23.2 |
| $Y_{PHA/Glu}$ (g/g) | 0.19 | 0.27 | 0.32 | 0.25 | 0.25 |

[a]Glucose (5 g/*l*) and valeric acid at varying concentrations were added when the culture reached an $OD_{600}$ of 1.3. Yeast extract (0.5 g/*l*) was added at the time of inoculation.
[b]Not detectable.

**Table 3. Effect of glucose concentration on P(3HB-co-3HV) synthesis[a]**

| | Glucose concentration (g/*l*) | | | | |
|---|---|---|---|---|---|
| | 0 | 2 | 5 | 10 | 20 |
| DCW (g/*l*) | 1.96 | 2.56 | 3.21 | 2.96 | 2.49 |
| PHA (g/*l*) | 0.27 | 0.79 | 1.56 | 1.28 | 1.35 |
| PHA content (wt %) | 13.8 | 30.9 | 48.6 | 43.2 | 54.2 |
| 3HV fraction (mol %) | 33.3 | 20.3 | 14.7 | 10.2 | 7.4 |
| $Y_{PHA/Glu}$ (g/g) | - | 0.40 | 0.32 | 0.13 | 0.11 |

[a]Glucose at varying concentrations and valeric acid (20 mM) were added when the culture reached an $OD_{600}$ of 1.3. Yeast extract (0.5 g/*l*) was added at the time of inoculation.

**2. Effect of Valeric Acid Concentration**

The effect of valeric acid concentration in the medium on 3HV fraction was investigated (Table 2). The 3HV fraction increased from 13.2 mol% at 10 mM valeric acid to 23.2 mol% at 40 mM valeric acid with an increase in valeric acid concentration. Total PHA accumulation reached a maximum of 1.56 g/*l* at 20 mM valeric acid, and decreased with further increase in valeric acid concentration. Cell mass and PHA yield on glucose ($Y_{PHA/GLU}$) were also the highest with 20 mM valeric acid. Copolymer production decreased with higher concentrations of valeric acid probably because of the toxic effect of valeric acid.

**3. Effect of Glucose Concentration**

The effect of glucose concentration on the copolymer synthesis was investigated. Both cell mass and PHA concentration reached a maximum at 5 g/*l* glucose. This result was different from Slater et al. who reported that both PHA production and 3HV fraction in the copolymer increased with an increase in glucose concentration. At glucose concentration greater than 5 g/*l* $Y_{PHA/GLU}$ decreased rapidly. The 3HV fraction continuously decreased with an increase of glucose concentration unlike the results reported by Slater et al. In our system, this difference could possibly be explained by severe catabolite repression of glucose. Pauli et al. also reported that *fadR* mutant strain showed a strong catabolite repression. Other carbon sources such as gluconate, glyoxylate, succinate were examined for their ability to relieve catabolite repression. However, less P(3HB-co-3HV) was accumulated with these carbon sources (data not shown). The 3HV fraction increased to 12.2 mol% using the medium containing 10 g/*l* fructose, while the 3HV fraction of 6.9 mol% was obtained with 10 g/*l* glucose. With glucose as the carbon source, higher polymer concentration could

**Table 4. Effect of c-AMP and oleic acid on P(3HB-co-3HV) synthesis[a]**

| | Control | c-AMP[b] | Oleic acid[c] |
|---|---|---|---|
| DCW (g/l) | 2.42 | 2.24 | 2.81 |
| PHA (g/l) | 1.15 | 1.01 | 1.28 |
| PHA content (wt %) | 47.5 | 45.1 | 45.6 |
| 3HV fraction (mol %) | 6.9 | 7.9 | 19.5 |

[a]Glucose (10 g/l) and valeric acid (20 mM) were added when the culture reached an $OD_{600}$ of 0.8. Yeast extract (0.5 g/l) was added at the time of inoculation.
[b]c-AMP (100 mg/l) was added at an $OD_{600}$ of 0.8.
[c]Oleic acid (1 g/l) and Brij 35 (4 g/l) were added when the culture reached an $OD_{600}$ of 0.8.

**Table 5. Effect of yeast extract on P(3HB-co-3HV) synthesis[a]**

| | Yeast extract concentration (g/l) | | | |
|---|---|---|---|---|
| | 0 | 0.5 | 2 | 5 |
| DCW (g/l) | 2.18 | 2.37 | 1.52 | 1.64 |
| PHA (g/l) | 0.72 | 1.02 | 0.48 | 0.37 |
| PHA content (wt %) | 33.1 | 43.1 | 31.6 | 22.6 |
| 3HV fraction (mol %) | 10.3 | 6.3 | 4.2 | 5.4 |

[a]Glucose (15 g/l) and valeric acid (20 mM) were added when the culture reached an $OD_{600}$ of 0.8.

be obtained by delaying the glucose addition time.

**4. Relieving Catabolite Repression**

Glucose causes catabolite repression by lowering the intracellular level of cyclic adenosine 3',5'-monophosphate (cyclic AMP) which, in combination with a cyclic AMP receptor protein (CRP protein), is required for the maximal expression of the relevant genes [Pastan and Adhya, 1976]. It was reported that the strong repression of inducible enzyme synthesis involved in the fatty acid degradation could be partially relieved by the addition of cyclic AMP to the growth medium [Pauli et al., 1974]. However, in our study the 3HV fraction did not increase significantly with the addition of cyclic AMP (Table 4). It seems that the derepression of fatty acid degradation enzymes by cyclic AMP was not enough to increase the utilization of valeric acid.

The expression of fatty acid degradation enzymes is induced by a long-chain-length fatty acids like oleic acid [Weeks et al., 1969]. When 1 g/l of oleic acid was added to the glucose containing medium the 3HV fraction (19.5 mol%) increased threefold (Table 4).

**5. Effect of Nitrogen Sources**

PHA formation can be affected by the type of nitrogen source used [Lee and Chang, 1995]. Yeast extract was an effective nitrogen source for the formation of PHB by *Alcaligenes* sp. The effect of yeast extract on cell growth and P(3HB-co-3HV) accumulation was examined in flask cultures containing a defined medium supplemented with varying amounts of yeast extract (Table 5). Addition of a small amount of yeast extract (0.5 g/l) promoted P(3HB-co-3HV) synthesis. Further addition of yeast extract decreased copolymer production. Various nitrogen sources were also tested for their ability to promote P(3HB-co-3HV) synthesis (Table 6). Addition of 1 g/l tryptone and peptone resulted in the increase of P(3HB-co-3HV) concentration by two fold.

The addition of valine or isoleucine (0.1 g/l) increased the 3HV concentration in the copolymer [Yim, 1994]. This is probably because the level of propionyl-CoA, the precursor of 3HV monomer, was increased by the breakdown of valine and isoleucine.

**Table 6. Effect of various nitrogen sources on P(3HB-co-3HV) synthesis[a]**

| | Nitrogen source[b] | | | | |
|---|---|---|---|---|---|
| | Control | Yeast extract | Tryptone | Peptone | Casamino acids |
| DCW (g/l) | 1.70 | 2.00 | 2.37 | 2.60 | 2.20 |
| PHA (g/l) | 0.75 | 0.54 | 1.39 | 1.51 | 0.84 |
| PHA content (wt %) | 44.1 | 27.0 | 58.7 | 58.1 | 38.2 |
| 3HV fraction (mol %) | 13.3 | 9.3 | 8.7 | 10.6 | 9.5 |

[a]Glucose (5 g/l) and valeric acid (20 mM) were added when the culture reached an $OD_{600}$ of 1.3.
[b]All nitrogen sources (1 g/l) were added at the time of inoculation.

## CONCLUSIONS

In bio-synthesis of P(3HB-co-3HV) copolymer by recombinant *E. coli fadR atoC* mutant harboring the *A. eutrophus* PHA biosynthesis genes, P(3HB-co-3HV) synthesis increased by reducing acetic acid formation. Less acetic acid was formed by increasing the initial acetic acid concentration, which decreased the cell growth rate. At an initial acetic acid concentration of 50 mM with 20 g/l of glucose and 20 mM of valeric acid PHA accumulated most. The optimal level of valeric acid for the copolymer synthesis was 20 mM. We could regulate the ratio of 3HV to 3HB in the copolymer by; (1) varying glucose and valeric acid concentrations, (2) adding oleic acid, (3) decreasing the growth temperature. An addition of valine or isoleucine also increased the 3HV fraction since they may provide propionyl-CoA during the breakdown. Based on these findings, we are currently developing an efficient fermentation strategy to produce a high concentration of P(3HB-co-3HV) copolymer with high productivity by recombinant *E. coli*.

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