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Characterization of a highly attenuated *Salmonella enterica* serovar Typhimurium mutant strain

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Salmonella enterica serovar Typhimurium ATCC 13311 is virulent at a dose as low as 10^2 colony-forming units when administered intraperitoneally to BALB/c mice. In order to develop highly attenuated mutant strain through the combination of 2 phenotypically attenuated markers, we constructed a number of amino acid requiring auxotrophic strains of *S. enterica* serovar Typhimurium by means of UV-induced mutations. One of them, strain NDMC-B1, was highly attenuated for mice, with an LD_{50}-value of 6 and 3 log units lower for mice than the wild-type strain and *S. enterica* serovar Typhimurium *aroA* strain, respectively. This strain still contained the *Salmonella* O- and H-antigens but had a requirement for cysteine and was unable to utilize citrate as its sole carbon source. NDMC-B1 colonized the gut-associated lymphoid tissue more efficiently than the wild-type strain, but its capacities to colonize spleen and liver were significantly reduced. Mice intraperitoneally or orally vaccinated with NDMC-B1 were highly protected against either an intraperitoneal challenge with 10^6 colony-forming units or an oral challenge with 10^9 colony-forming units of the wild-type strain. Taken together, the results illustrate that through the combination of 2 independently phenotypical attenuating markers, the requirement for cysteine and the inability to use citrate, we have successfully constructed a highly attenuated, stable, and immunogenic *S. enterica* serovar Typhimurium vaccine strain which can induce protective immunity in a mouse model against lethal challenge of wild-type strain.

Key words: Attenuation, auxotrophic mutant, immunogenicity, *Salmonella enterica* serovar Typhimurium

The inactivated whole-cell *Salmonella* vaccine has long been used for preventing infection of salmonellosis; however, this vaccine has shown variable efficacy in controlled field trials and can cause serious side effects in some recipients [1-4]. In contrast, live attenuated *Salmonella* vaccines have been shown to confer greater and longer-lasting immunity in mice than inactivated vaccines [5,6]. Thus, many attempts to develop live oral vaccines against typhoid have been reported. Since the delivery of antigens to the gut-associated lymphoid tissue (GALT) can lead to generalized humoral and cellular as well as secretory immune responses [7-11], the use of live attenuated *Salmonella* organisms as an oral vaccine has an apparent advantage in that *Salmonella* spp. can initially colonize the GALT prior to inhabiting deeper tissues [12].

Several methods have been used to obtain an attenuated strain of *Salmonella* spp. without impairing immunogenicity, including mutations altering synthesis of amino acids and gene expression [13,14]. For example, *aroA*-deletion mutants of *Salmonella enterica* serovar Typhimurium (*S.* serovar Typhimurium) were found to be avirulent and immunogenic in mice and were excellent live oral vaccines against murine salmonellosis [15-18]. However, attenuation by only one single or 2 related phenotypical markers does not provide sufficient safety against vaccination-related complications. Practically, 2 independently phenotypical attenuating markers are necessary to ensure full stability on account of potentiating single frequencies of backmutation [19,20] although certain one-marker mutants exhibit good stability. In this study, we used UV-induced mutagenesis to construct a stable, highly attenuated, and immunogenic *Salmonella* vaccine strain NDMC-B1 through the combination of 2 independently phenotypical attenuating markers, the requirement for cysteine and the inability to use citrate as a carbon source.

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Materials and Methods

Bacterial strains and media

S. serovar Typhimurium ATCC 13311 and *S.* serovar Typhimurium *aroA* strain ATCC 33275 were obtained from the American Type Collection Center. Bacteria were routinely cultured on Luria media [21] and maintained in 1% Bacto Peptone (Difco Laboratories, Detroit, MI, US) containing 5% glycerol for storage in duplicate at –70°C. M9 minimal medium containing 0.1% NH_4Cl, 0.6% Na_2HPO_4, 0.3% KH_2PO_4, 0.05% NaCl, 0.001% $CaCl_2$, 0.02% $MgSO_4$, 0.2% glucose, and 1.5% agar, pH 7.4 [21], and M9 minimal medium supplemented with various growth factors at the appropriate concentration [22] were used for identifying auxotrophic mutant. Solid media contained 1.5% (w/v) agar (Difco).

Mutagenesis and auxanography

Initially, 100 μL culture broth containing 10^9 colony-forming units (CFU)/mL of *S.* serovar Typhimurium ATCC 13311 grown in Luria broth at 37°C for 12 h was spread on the Luria agar plate. These plates were irradiated with UV light at a dose of 400 J/m^2 and then incubated at 37°C for 3 days. Auxotrophic mutants were selected from those irradiated survivors that were unable to grow on M9 minimal medium plate. The auxanography was carried out according to the method of Holliday [23], as slightly modified by Liu [22] using pools of growth factors on agar plates. Briefly, 36 possible requirements were arranged for testing the single growth factor. Each of 12 M9 minimal medium plates was supplemented with different combinations of 6 factors with the arrangement as previously described [22]. The concentrations of amino acid, pyrimidine or purine, and vitamins (Sigma, St. Louis, MO, US) in the plate were 1 mM, 0.1 mM, and 0.01 mM, respectively. All the auxotrophic mutants were inoculated in all of the 12 M9 agar plates supplemented with growth factors and incubated at 37°C for 3 days. Cell growth was observed periodically during the incubation.

Characterization of NDMC-B1 strain

The physiological characteristics of NDMC-B1 strain and its parent strain were tested using an Enterotube II (Becton Dickinson Diagnostic Instrument System, Sparks, MD, US) system as directed by the manufacturer. The tube comprised several compartments for media, which allowed testing for the following physiological characteristics: utilization of monosaccharide and citrate as carbon source, decarboxylation of lysine and ornithine, and production of H_2S. Organisms growing overnight on a MacConkey agar plate were picked up with the end of a needle, and the media were inoculated by withdrawing the needle through the compartments. After overnight incubation of organisms, the results were read against a color chart. All the mutant strains were routinely tested for serologic characteristics with anti-H and anti-O diagnostic sera purchased from Becton Dickinson.

Growth utilizing various carbon sources

Studies on the growth of strains ATCC 13311 and NDMC-B1 utilizing various carbon sources were carried out according to the method described by Utely *et al* [24]. Generally, bacteria grown overnight in Luria broth at 37°C with aeration to about 1 x 10^9 CFU/mL were washed twice in M9 minimal medium lacking a carbon source and were resuspended in the same medium at about 10^9 CFU/mL. The cultures were diluted to about 10^5 CFU/mL in M9 minimal medium supplemented with either glucose (0.2%), sodium citrate (0.2%), lactose (0.2%), arabinose (0.2%), or sorbitol (0.2%). All cultures were grown for 24 h at 37°C with aeration and were then plated on Luria agar for viable counts. Plates were incubated at 37°C for 18 to 24 h prior to counting. The growth of the wild type and NDMC-B1 strain in Luria broth were determined by culturing the bacteria at 37°C with aeration and measuring the OD_{600} of the cultures at different incubation period.

Animal infections and enumeration of viable *S. enterica* serovar Typhimurium in mice

Innately *Salmonella*-susceptible BALB/c mice of 8 weeks of age, bred in the animal unit at the Animal Center of the National Science Council of the Republic of China, were used throughout. For virulence assays via the intraperitoneal route, inocula ranging from 50 CFU to 10^{10} CFU were injected by syringe in 100 μL of buffered saline with gelatin (BSG) with 8 mice in each group. For oral inoculation, bacteria were administered in 100 μL volumes to lightly ether-anesthetized mice by gavage as described by Maskell *et al* [18]. Deaths were recorded over the following 30 days. The 50% intraperitoneally lethal dose (LD_{50}) was calculated by the method of Reed and Muench [25]. To determine CFU in Peyer's patch, liver, and spleen, groups of 4 mice were orally inoculated with 10^9 CFU of the strain and necropsied on Days 1, 3, 7, 10, and 14 after inoculation. Necropsy procedures were as described by Curtiss and Kelly [26]. *S.* serovar Typhimurium ATCC 13311 was used as a wild-type control.

Peyer's patches of the intestinal tract, spleen, and liver were removed, weighed, and homogenized in Luria broth prior to death due to disease [27]. Homogenates (0.1 g/mL) were diluted and plated on MaConkey agar (Difco) supplemented with 1% (w/v) glucose to determine CFU per organ. Plates were incubated at 37°C for 18 h prior to counting. Data on CFU are presented as geometric means with standard errors.

Immunization and challenge experiments

Eight-week-old female BALB/c mice were used for either oral or intraperitoneal immunization experiments. The animal room was maintained at 25°C with 12 h of illumination daily. The strain NDMC-B1 was grown to late log phase in Luria broth at 37°C with aeration. For oral immunization, mice were deprived of food and water for 4 h prior to immunization and then were given 30 μL of sodium bicarbonate (10%, w/v) to neutralize stomach acid. Twenty minutes later, 100 μL of *S.* serovar Typhimurium cells suspended in BSG diluted to the desired density was administered through a pipette tip to the back of the mouth. Food and water were returned 30 min after inoculation. For intraperitoneal immunization, 100 μL of *S.* serovar Typhimurium cells suspended in BSG diluted to the desired density was administered by intraperitoneal injection. Thirty days post-immunization, all mice were intraperitoneally challenged with the wild-type strain. Mortality and morbidity of the challenged mice were observed for an additional 30 days.

Statistical analysis

The abilities of NDMC-B1 and the wild-type strain to colonize Peyer's patch and deeper tissues, such as spleen and liver, of BALB/c mice after oral infection were compared by using the unpaired Student's *t* test, as described elsewhere [28]. Statistical significance was assessed at *p* values of less than 0.01.

Results

Attenuation of virulent *S. enterica* serovar Typhimurium strain

Among 520 irradiated survivors obtained from the UV-induced mutagenesis experiment, 4 auxotrophic mutants derived from the wild type were isolated. The virulence of the wild type and the auxotrophic mutant strains were determined by intraperitoneal inoculation of mice with various doses of bacteria. The results in Table 1 showed that all auxotrophic mutants had a higher LD_{50} than the wild-type strain. Among them, mutant strain NDMC-B1 was markedly more attenuated than the other auxotrophic mutants, including NDMC-A1, A2, A3, and the *aroA* mutant strain ATCC 33275. Strain NDMC-B1 had a LD_{50} more than 10^6 and 10^3 times higher than those of the wild-type and *aroA* strain, respectively (Table 1), and did not kill the mice even when a dose of as high as 10^{10} organisms was given orally (data not shown). The abilities of attenuated and virulent strains to colonize Peyer's patch and the deeper tissue, such as liver and spleen, of BALB/c mice after oral infection were determined. NDMC-B1 strain appeared to colonize the Peyer's patch as efficiently as the wild-type strain for 7 days after infection ($p>0.05$), and yet its ability to colonize the spleen and the liver were significantly reduced as compared with that of the wild-type strain ($p<0.01$) (Fig. 1).

Protection of mice after intraperitoneal and oral challenge

Mice, either intraperitoneally or orally immunized with the NDMC-B1 strain, were challenged either intraperitoneally or orally with wild-type virulent salmonellae to determine the level of protection induced by the strain. At a single intraperitoneal dose of 2.5×10^5 CFU or greater, NDMC-B1 strain proved to be protective upon intraperitoneal challenge 30 days later with 8.8×10^6 CFU of the wild-type strain, that is, all vaccinated mice appeared completely healthy (Table

Table 1. Mortality of BALB/c mice 30 days after intraperitoneal inoculation with auxotrophic mutants of *S. enterica* serovar Typhimurium ATCC 13311

Strain	LD_{50} (CFU)[a]
S. enterica serovar Typhimurium ATCC 13311	4.4×10^2
Mutant NDMC-A1	1.6×10^3
Mutant NDMC-A2	1.8×10^4
Mutant NDMC-A3	2.5×10^5
Mutant NDM C-B1	7.5×10^8
S. enterica serovar Typhimurium *aroA* strain ATCC 33275	3.5×10^5

Abbreviations: LD_{50} = 50% of lethal dose; CFU = colony-forming unit

[a]Mice were intraperitoneally inoculated with the indicated strains. Mortality was observed for 30 days.

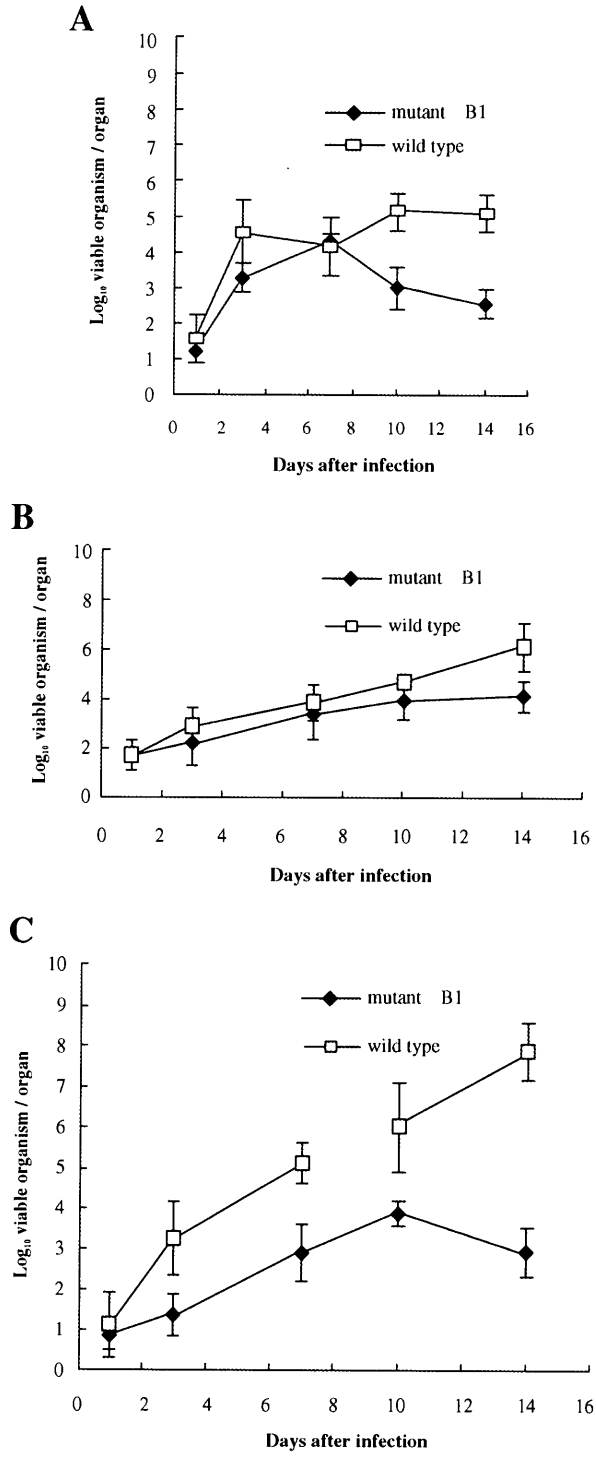

Fig. 1. Recovery of the mutant strain NDMC-B1 and the wild-type strain from **(A)** Peyer's patch, **(B)** spleen, and **(C)** liver of 8-week-old BALB/c mice at specified times after oral infection with 10^9 CFU. Each point represents the geometric mean ± standard error.

2). In contrast, age-matched non-vaccinated BALB/c mice challenged with the same dose of the wild-type strain all died within 8 days (Table 2). In addition, oral immunization with 3.7×10^8 CFU of NDMC-B1 strain or greater also elicited an adequate protection against oral challenge with 8.8×10^9 CFU of the wild-type strain on Day 30 postimmunization (Table 3).

Auxanographic analysis

The results of auxanographic analysis indicated that strain NDMC-B1 grew very poorly in plates no. 1, 2, 3, 5, 6, 7, 9, 10, 11, and 12, whereas the cells in plates no. 4 and 8 grew very quickly (data not shown). These data suggest that cysteine, the growth factor existing in both plates, may be the essential nutrient required for the growth of strain NDMC-B1.

Physiological characteristics of strain NDMC-B1

NDMC-B1 and the wild-type strains were subjected to 11 biochemical tests, and the physiological properties of strain NDMC-B1, which are different from those of the wild-type strain, are summarized in Table 4. Strain NDMC-B1 was unable to decarboxylate lysine and ornithine, produce H_2S, ferment arabinose, sorbitol, and dulcitol, or to utilize citrate as a carbon source. However, both NDMC-B1 and the wild-type strain agglutinated with *Salmonella* O- and H-antiserum (data not shown), indicating that they are identical with respect to O- and H-antigens. In addition to being unable to utilize citrate, NDMC-B1 strain was unable to utilize arabinose and sorbitol as sole carbon sources for growth (Table 5).

Although NDMC-B1 could utilize glucose as carbon source for growth, it grew very slowly in Luria broth at 37°C and to only one-fourth the final yield of the wild-type strain (Fig. 2).

Genetic stability of inability to utilize citrate in NDMC-B1

Strain NDMC-B1 was grown overnight at 37°C with aeration in Luria broth. After washing the culture in M9 minimal medium, the cells were suspended in M9 broth at the concentration of 5×10^9 CFU/mL, and then 100 µL aliquots were spread on M9 agar plates containing sodium citrate (0.2%) as the sole carbon source. The plates were incubated for 36 h at 37°C. We did not find a single colony that was able to utilize citrate as a carbon source among 5×10^8 CFU plated.

Discussion

The results of this study demonstrated that the UV-induced auxotrophic mutation attenuated the virulent *S.* serovar Typhimurium ATCC 13311. Of the auxotrophic mutants, strain NDMC-B1 appeared to be more

Table 2. Effectiveness of single intraperitoneal immunization with mutant strain NDMC-B1 in protecting BALB/c mice against intraperitoneal challenge with virulent wild-type strain

Immunizing dose (CFU)	Challenging dose (CFU)	No. of survivors/total no. of mice[a]
2.5×10^7	4.4×10^6	6/6
2.5×10^6	4.4×10^6	6/6
2.5×10^5	8.8×10^6	6/6
2.5×10^4	8.8×10^6	4/6
PBS[b]	8.8×10^6	0/6

Abbreviations: CFU = colony-forming unit; PBS = phosphate buffer solution

[a]Thirty days after mice were immunized intraperitoneally with a single dose of the NDMC-B1 strain, they were challenged with wild-type strain. Morbidity and mortality observations were recorded daily for an additional 30 days postchallenge.

[b]Phosphate buffer solution was used instead of bacterial suspension as a negative control.

Table 3. Effectiveness of single oral immunization with attenuated mutant strain NDMC-B1 in protecting BALB/c mice against oral challenge with virulent wild-type strain

Immunizing dose (CFU)	Challenging dose (CFU)	No. of survivors/total no. of mice[a]
3.7×10^{10}	4.4×10^9	6/6
3.7×10^9	4.4×10^9	6/6
3.7×10^8	8.8×10^9	6/6
3.7×10^7	8.8×10^9	5/6
PBS[b]	8.8×10^9	0/6

Abbreviations: CFU = colony-forming unit; PBS = phosphate buffer solution

[a]Thirty days after mice were immunized orally with a single dose of NDMC-B1 strain, they were challenged with wild-type strain. Morbidity and mortality observations were recorded daily for an additional 30 days postchallenge.

[b]Phosphate buffer solution was used instead of bacterial suspension as a negative control.

attenuated than either the wild-type or the *aroA* mutant strain, with an LD_{50}-value of 6 and 3 log units lower for mice than the wild type and *aroA* mutant strain [15], respectively. Even the mice that orally received greater than 10^{10} CFU of NDMC-B1 strain did not develop any visible signs of illness. However, strain NDMC-B1 colonized the GALT as efficiently as the wild-type strain although its capacities to colonize the spleen and liver were significantly reduced. Since *S.* serovar Typhimurium initiate infection in the terminal ileum by invading M-cells, the surrounding enterocytes of the Peyer's patches, and then the underlying lymphoid cells [29-30], the data presented here suggest that the auxotrophic mutation of strain NDMC-B1 did not interfere with its ability to invade, grow, or survive in M-cells, enterocytes, and the underlying lymphoid cells as compared with the wild-type strain. Furthermore, NDMC-B1 is much more efficient at colonizing GALT than internal lymphoid organs, suggesting that such an attenuated strain may be more effective in inducing generalized mucosal immune responses than inducing either systemic or cellular immune response [31].

The data on protection obtained with intraperitoneal or oral challenge of mice strongly suggest that strain NDMC-B1 can be used as a live oral vaccine candidate. All mice intraperitoneally or orally immunized with a single dose of NDMC-B1 were protected against intraperitoneal challenge with 2×10^4 times the LD_{50} or oral challenge with 10^9 CFU of the wild-type strain, respectively.

In contrast to the wild-type, NDMC-B1 possesses an auxotrophic requirement for cysteine and lacked

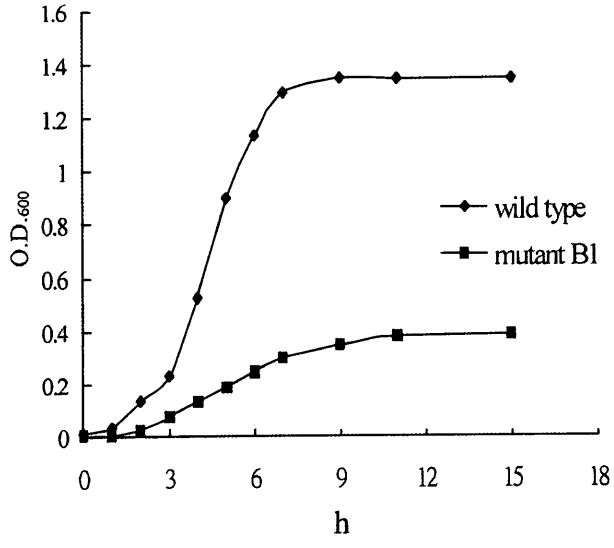

Fig. 2. Growth curves of mutant strain NDMC-B1 and the wild-type strain in Luria broth at 37°C.

Table 4. Physiological characteristics of NDMC-B1 and the wild-type strain

Biochemical reaction	Characteristic[a]	
	Wild-type	NDMC-B1
Fermentation of glucose	+	+
Fermentation of adonitol	–	–
Fermentation of lactose	–	–
Fermentation of dulcitol	+	–
Fermentation of arabinose	+	–
Fermentation of sorbitol	+	–
Decarboxylation of lysine	+	–
Decarboxylation of ornithine	+	–
Production of H_2S	+	–
Hydrolization of urea	–	–
Utilization of citrate	+	–

[a] "+" means positive reaction; "–" means negative reaction.

several phenotypes including the ability to utilize citrate, to ferment arabinose and sorbitol, to decarboxylate lysine and ornithine, and to produce H_2S. These phenotypic characteristics could be used as markers to differentiate the vaccine strain from other isolates in the field. In a stepwise series of oxidative reactions, citrate is converted back to oxaloacetate in the tricarboxylic acid (TCA) cycle, which allows the organism to generate substantially more energy per mole of glucose than is possible with glycolysis alone.

Thus, failure to further utilize citrate may result in limited generation of energy essential for growth due to incomplete oxidation of glucose. In addition, some activity of the TCA cycle is required since α-ketoglutarate and succinyl-CoA are required for amino acid biosynthesis in a minimal medium [32]. This result is consistent with the observation of Utley *et al* [24] that the phenotype unable to use citrate may be related to the attenuation of *S.* serovar Typhimurium. With the exception of failure to synthesize cysteine and to utilize citrate, strain NDMC-B1 and the wild-type strain were identical with respect to O- and H-antigens, suggesting that such auxotrophic mutation did not interfere with the synthesis of flagella and cell wall [33].

In this investigation, we have successfully generated a highly attenuated, stable, and immunogenic *S.* serovar Typhimurium vaccine strain through the combination of 2 independently phenotypical attenuating markers that are unable to synthesize cysteine or use citrate as a sole carbon source. This observation may have an important impact on the design of attenuated *S.* serovar Typhi strains with multiple genetic defects in synthesizing amino acid and using citrate as live oral typhoid vaccines.

Acknowledgments

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Table 5. Carbon sources utilized by NDMC-B1 and the wild-type strain

Carbon source	Wild-type (24 h CFU/input CFU)[a]	NDMC-B1(24 h CFU/input CFU)
None	2.5	1.2
Glucose	882	356
Citrate	386	0.8
Arabinose	428	1.8
Lactose	24	2.1
Sorbitol	265	2.0

Abbreviation: CFU = colony-forming unit

[a]Input CFU/mL and 24 h CFU/mL were defined as the cell concentration in the M9 minimal medium supplemented with different carbon sources before and after 24 h incubation, respectively.

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