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Development of an Improved Selective Agar Medium for Isolation of *Yersinia pestis*

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Existing media designed for selective isolation of clinically important members of the genus *Yersinia* were found to be unsatisfactory for the growth and isolation of *Yersinia pestis*. We report the development of a new selective agar medium (termed BIN) that supports the growth of *Y. pestis*. The development of the formulation of this medium was based on a fluorescence screening system designed for monitoring bacterial growth on semisolid media, using a green fluorescent protein-expressing strain. High-throughput combinatorial experiments can be conducted for the quantitative evaluation of the effect of different medium components on growth. Generation of fluorescence plots in this system, using microplates, allowed the quantitative evaluation of the growth rate of *Y. pestis* EV76 cultures in different agar compositions. The final BIN formulation is based on brain heart infusion agar, to which the selective agents irgasan, cholate salts, crystal violet, and nystatin were introduced. It was found that BIN agar is more efficient in supporting colony formation and recovery of *Y. pestis* than are the conventional semisolid media MacConkey agar and *Yersinia*-selective agar (cefsulodin-irgasan-novobiocin agar). The advantage of BIN over other media has been also demonstrated in recovering virulent *Y. pestis* from the mixed bacterial populations found in decaying carcasses of infected mice. The BIN medium is suggested as a selective medium for isolation and recovery of *Y. pestis* from various backgrounds.

Plague, caused by the bacterial pathogen *Yersinia pestis*, is one of the oldest recorded infectious diseases and in the past has claimed millions of lives (6, 20, 21). Natural foci of plague are still spread worldwide, mainly in the rodent and flea vector reservoirs (26, 27). Periodic epizootics, climate changes, rodent population size and migration, and landscape modifications affect the dynamics of most plague focus outspreads. Most human plague cases in recent years occurred among individuals who were in contact with wild rodents in areas of endemicity (6), but occasionally infections were reported in areas where *Y. pestis* infected domestic carriers such as rats, cats, and several other mammalian species (7, 12). The thousands of cases reported yearly to the World Health Organization (WHO) in the last decade indicate that plague is far from being eradicated and continues to present a threat, especially where public health and living conditions are poor (26). Future global pandemics are improbable; however, the potential use of *Y. pestis* as a biological weapon could cause pneumonic plague in large populations (14).

The course of clinical infection is often characterized by fast progression in vivo, and death occurs rapidly if proper antibiotic treatment is not applied within 18 to 24 h of disease onset (4, 5, 21). Unlike most *Enterobacteriaceae*, all known *Y. pestis* strains exhibit slow growth in vitro on conventional laboratory media (3, 23). Thus, development of a medium that would facilitate rapid yet specific isolation of *Y. pestis* may have an important impact on both clinical diagnosis and plague surveillance.

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Current WHO regulations for isolation of *Y. pestis* recommend the use of brain heart infusion agar (BHIA), sheep blood agar, and MacConkey agar (10). These growth media are best suited for isolation of the bacteria from clinical samples that are otherwise usually sterile (such as blood, lymph node, bubo aspirates, and cerebrospinal fluid), so that the infectious agent is expected to grow as a pure culture. However, when the clinical form of disease requires the use of nonsterile samples such as sputum, respiratory tract swabs or washings, skin swabs, or skin scrapings, the isolation of *Y. pestis* may be complicated by the presence of background flora competing for nutrients and resources of the medium (especially on the nonselective rich media), due to both higher growth rates and possibly higher initial numbers. This problem becomes more pronounced when environmental samples or decomposing carcasses of infected animals are tested for the presence of *Y. pestis* (16).

Among the media recommended by the WHO for *Y. pestis* isolation, only MacConkey agar possesses a certain degree of selectivity, mainly due to the presence of crystal violet (which inhibits gram-positive organisms) and bile salts (which inhibit growth of nonenteric bacteria). However, since MacConkey medium was originally developed for isolation of enteric bacteria in general, it allows growth of a large number of gram-negative organisms. Moreover, the slow growth exhibited by *Y. pestis* on this medium restricts its applicability as a selective medium for mixed cultures. Few attempts have been made in the past to develop selective and/or differential media for *Y. pestis* (11, 17, 18, 19, 25). More recently, cefsulodin-irgasan-novobiocin (CIN) agar, developed primarily for isolation of *Yersinia enterocolitica* (24), has been proposed as an alternative selective medium for *Y. pestis* (22). Although *Y. pestis* can generally tolerate the levels of selective substances used in CIN

agar, only a portion of the plated bacteria grow to form colonies (1, 23). It thus appears that the formulations of selective agents at the levels used in CIN and MacConkey agars cause a decrease in growth rate that results in a lower recovery of *Y. pestis*.

In the present report, we document the formulation of a novel improved medium that has the high selectivity required for isolation of *Y. pestis* from a variety of sources. Furthermore, the new medium enables a significantly improved recovery of *Y. pestis* compared to the presently available selective media.

MATERIALS AND METHODS

Media. Luria-Bertani broth, BHIA, CIN medium, and MacConkey agar (all from Difco) were prepared according to instructions of the manufacturer (Difco manual, 11th ed., 1998). The individual components of CIN medium used for preparation of basal CIN medium (growth-supportive components of CIN medium without the selective agents [see Table 2]) were from Difco, except for D-mannitol (Sigma) and sodium chloride (Merck). Blood agar plates supplemented with 5% defibrinated sheep blood were purchased from a local supplier (Hy-Laboratories). Stock solutions used for the formulation of different medium combinations were prepared as follows. Irgasan DP300 (Ciba-Geigy) solution was made at 0.2 mg/ml in 90% ethanol and was added aseptically to autoclaved media after cooling to about 80 to 85°C, followed by vigorous shaking to volatilize the ethanol. Crystal violet stock solution was prepared at 1 mg/ml in double-distilled water (ddH_2O) and autoclaved. Sodium cholate and sodium deoxycholate (both from Sigma) were prepared by dissolving 5 g each in 100 ml of ddH_2O and autoclaved. Nystatin (Sigma) (10^5 U/ml) was prepared in ddH_2O and vigorously mixed by vortexing before use. Cefsulodin (1.5 mg/ml) and novobiocin (1.5 mg/ml) solutions (both from Sigma) were stored at −70°C and thawed just before use. All media were poured either at 25-ml volumes in standard petri dishes or at 1-ml volumes in 24-well (flat-bottom) tissue culture plates (Techmo Plastic Products).

Bacterial strains. *Yersinia* strains included the virulent *Y. pestis* Kimberley53 (2, 13); the nonvirulent *Y. pestis* TRU, A1122, and EV76 (2); and the enteric *Y. enterocolitica* WA (ATCC 27729), *Y. enterocolitica* CDC 497-70 (ATCC 29913), and *Yersinia pseudotuberculosis* III and 134 C strains (2). Other gram-negative strains used in selectivity studies were *Escherichia coli* ATCC 25922 and ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212 and ATCC 51299, and local isolates of *Salmonella enterica* serovar Typhimurium, *Vibrio cholerae*, *Shigella dysenteriae*, and *Serratia marcescens*. Gram-positive strains used were *Staphylococcus aureus* ATCC 25923 and ATCC 29213, *Bacillus cereus* ATCC 10987 and ATCC 14579, *Bacillus thuringiensis* subsp. *israelensis* ATCC 35646, *Bacillus amyloliquefaciens* 10A1 (Bacillus Genetic Stock Center), *Bacillus subtilis* WB600 (28), and *Bacillus anthracis* Δ14185 (8). The strains were kept frozen at −70°C. For each experiment, bacteria were plated for isolation on BHIA and incubated for 1 day at 28°C for the enteric *Yersinia* strains, for 2 days at 28°C for *Y. pestis* strains, or for 1 day at 37°C for all other strains. Comparative studies with different medium formulations were conducted at 28°C. Plates were examined after 24, 36, and 48 h of incubation (and in some cases after up to 4 days) for CFU counts, colony size, and color.

Plating efficiency tests. The ability of different media to support the growth and recovery of *Y. pestis* was determined by the efficiency of colony formation. Bacterial suspensions from fresh colonies grown on BHIA were adjusted in sterile phosphate-buffered saline (PBS) to optical density at 660 nm (OD_{660}) of 0.2 (ca. 1×10^8 to 3×10^8 CFU/ml). The bacterial suspensions were serially diluted 10-fold, and 0.1-ml aliquots of each dilution were plated on at least triplicate plates of the tested media. The recovery percentage was calculated from the ratio of the mean counts of colonies on the test medium and on nonselective BHIA as a reference. The size of colonies at a given incubation period was also used to evaluate the effect of selective substances on growth.

Construction of *Y. pestis* strain EV76 expressing GFP. The pGFPuv plasmid (Clontech Laboratories Inc.) was electroporated into competent EV76 cells essentially as described by Conchas and Carniel (9). In short, cells were cultured in Luria-Bertani medium (to an OD_{660} of 0.7), washed twice in cold ddH2O, and concentrated to 4×10^9 CFU/ml in transformation buffer (15% glycerol, 0.27 M sucrose), and 0.1 ml was electroporated with 250 ng of plasmid DNA. The transformation efficiency was 5.5×10^5 CFU/μg. Colonies expressing GFP were clearly visible by UV illumination. Several clones were picked at random, and the presence of the three *Y. pestis* virulence plasmids was confirmed. The fluorescence levels (excitation wavelength, 410 nm; emission wavelength, 510 nm) emitted by bacterial suspensions of the derivative strain EV76(pGFPuv) were found to correlate directly with the CFU counts. Other growth characteristics of these clones were not different from those of the progenitor strain. The pGFPuv plasmid was found to be very stable in *Y. pestis* even after many passages without antibiotic selection.

Evaluation of growth rate on semisolid media. The effect on growth of the supportive and/or selective substances that were incorporated into different agar formulations was evaluated by using the GFP-expressing EV76 strain. For this purpose, a bacterial suspension in PBS (OD_{660} of 0.02) was spread in aliquots of 0.03 ml on the surface of the tested medium, which was previously prepared in a 24-well microplate (3×10^5 to 1×10^6 CFU/well). The microplates were incubated in a spectrofluorimeter (SPECTRAFluor Plus; Tecan) at 28°C, and the fluorescence emitted by the bacteria was determined hourly. For each culture, relative fluorescence units were plotted against incubation time, using the fluorescence values at the beginning of the incubation as background. At least six replicates were tested for each medium combination.

Selectivity tests. Tested strains were isolated on BHIA, and several fresh colonies were used to prepare suspensions containing ~10^8 CFU/ml in PBS. These suspensions were serially diluted 10-fold, and aliquots of 10 μl from each dilution were placed on BHIA, BIN agar, MacConkey agar, and CIN agar. Isolation of different strains on the tested media was conducted with standard 1-μl bacterial loops from concentrated colony suspensions (>10^9 CFU/ml). Selectivity tests were conducted at 28°C and monitored daily for 3 days.

Recovery from infected mice. Female mice (6 weeks old, OF1 outbred; purchased from Charles River Laboratories, Saint Aubin les Elbeuf, France) were inoculated subcutaneously with 100 50% lethal doses (LD_{50}) of the virulent *Y. pestis* Kimberley53 strain (1 LD_{50} = 1 CFU). Dead mice were kept separately at room temperature, and after 3, 5, 8, and 11 days their spleens and livers were homogenized separately in 2 ml of PBS. Samples were prepared from at least five corpses at each time point. Each sample was streaked for isolation and was also serially diluted 10-fold in PBS for comparative *Y. pestis* isolation and recovery efficiency tests on several semisolid media (BHIA, BIN agar, CIN agar, MacConkey agar, and blood agar). The plates were incubated at 28°C for up to 3 days, and growth was recorded daily for CFU counts, colony size, and color. Colonies isolated from BIN and CIN media were further verified as *Y. pestis* by immunofluorescence staining with polyclonal rabbit anti-F1 antibodies, as previously described (13). These experiments were conducted in compliance with Israeli law and the Israel Institute for Biological Research ethical policies for use of experimental animals.

TABLE 1. Recovery of *Yersinia* on commercially available selective agar media

Species and strain	% Plating efficiency (Mean ± SD) compared to BHIA[a]		
	Basal CIN agar[b]	CIN agar	MacConkey agar
Y. pestis EV76	108 ± 9	5 ± 2[c]	38 ± 15[c]
Y. enterocolitica CDC 497-70	104 ± 12	100 ± 5	98 ± 5

[a] CFU were determined after 36 h of incubation at 28°C.
[b] Formulation of growth-supportive components of CIN only.
[c] Wide range of colony size.

RESULTS AND DISCUSSION

Growth of *Y. pestis* on commercial selective semisolid media. The problematic growth of *Y. pestis* compared to that of *Y. enterocolitica* is exemplified on the commercially available selective media MacConkey agar and CIN agar and the nonselective medium basal CIN agar (containing only the growth-supportive components of CIN agar) and BHIA (Table 1). As expected, *Y. enterocolitica* demonstrated high recoverability on the tested media. However, *Y. pestis* strain EV76 yielded high recovery levels and homogeneous colonies (in terms of size range) only on the nonselective media, while plating on the selective media resulted in reduced growth. Colonies of EV76 grown on MacConkey agar exhibited a wide range of sizes, with

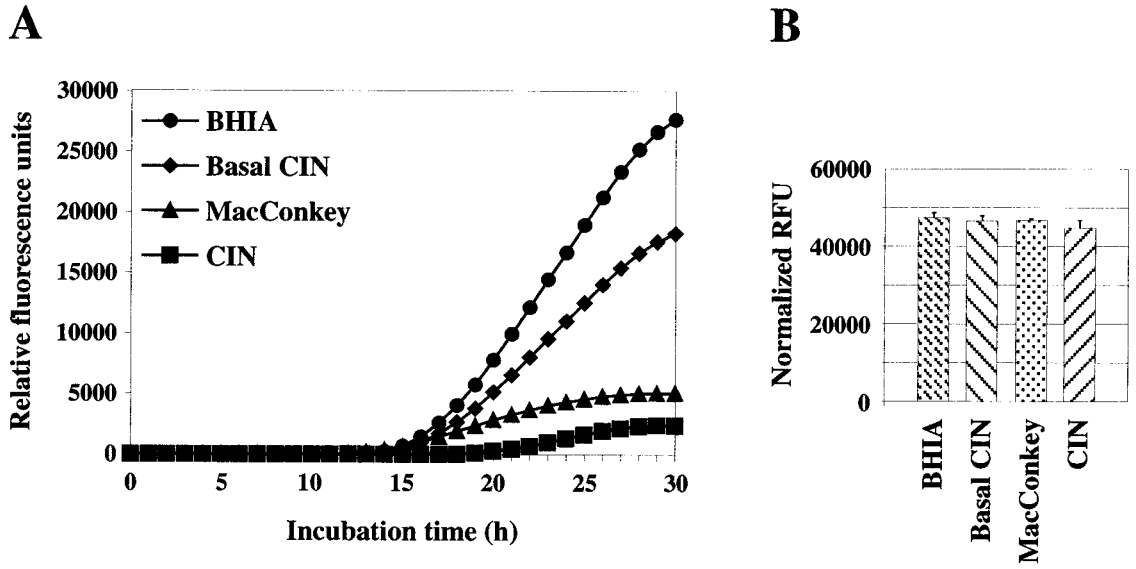

FIG. 1. Growth of the GFP-expressing *Y. pestis* EV76(pGFPuv) strain on commercially available semisolid media. (A) Fluorescence emission curves of agar cultures on the indicated media, incubated at 28°C. (B) Fluorescence of bacterial suspensions containing 10^8 CFU/ml in PBS prepared from the indicated agar cultures. RFU, relative fluorescence units.

all of the colonies being smaller than those developing in the enriched media. The percentages of recovery of EV76 (Table 1) were dependent on the incubation period, and higher values (90 to 100%) were obtained after prolonged incubation for up to 4 days (data not shown). CIN medium was found to be more restrictive than MacConkey agar for the growth of *Y. pestis*. Only 5% of the bacteria were recovered after 36 h, and the colonies were in a wide range of sizes. CFU counts were consistently lower by about 1 order of magnitude than those on the nonselective media, and they did not increase upon prolonged incubation. Our results are in agreement with the quantitatively reduced growth of *Y. pestis* on CIN agar reported previously by Russell et al. (23). The differences in growth between the nonselective and selective media may be attributed to a lack of proper growth-supportive nutrients and/or to the presence of inhibitory substances or selective agents in MacConkey and CIN agars that restrict the growth of *Y. pestis*.

Formulation of a new selective medium for *Y. pestis*. For the evaluation of the effect of medium components on growth of *Y. pestis*, we monitored the growth on semisolid media. For this purpose, a *Y. pestis* strain constitutively expressing GFP, EV76(pGFPuv), was constructed. The bacteria were grown in agar-containing microplate wells and incubated in a fluorescence plate reader. A direct correlation between the fluorescence level and bacterial cell mass was found by quantifying both the fluorescence and the CFU present in bacterial suspensions prepared from these agar cultures, as demonstrated in Fig. 1. This correlation allowed the determination of the growth rate on agar by monitoring (hourly) the fluorescence emitted by the bacterial culture in situ during incubation. Fluorescence-derived growth curves on BHIA were used as the reference for optimal growth rate. As shown in Fig. 1, the growth rate on CIN and MacConkey agars is lower than that on BHIA. Thus, low recoverability of *Y. pestis* on these media, as shown by colony formation efficiency, was manifested also by the slow accumulation of cell mass during incubation, using monitoring of fluorescence emitted by growing bacteria (Fig. 1). Furthermore, this type of evaluation showed that the basal CIN formulation is less growth supportive than the BHIA. For this reason, BHIA was chosen as the basal medium for the formulation of an optimized combination of selective agents that would support high recoverability of *Y. pestis*. The growth inhibition effect of various selective agents was quantified by introducing them individually into BHIA at several concentrations, and the growth rates on the different agar formulations were monitored by using the GFP reporter. This strategy allowed the determination of the maximal levels of irgasan (1 μg/ml), crystal violet (0.5 μg/ml), and bile salts (sodium cholate and sodium deoxycholate, 500 μg/ml each) that are tolerated by EV76 with no inhibition effect on the growth rate (Fig. 2). Both cefsulodin and novobiocin were clearly harmful and hampered growth at the concentrations routinely used in CIN agar (4 and 2.5 μg/ml, respectively). As expected, the mycobacterial agent nystatin showed no effect on growth of the reporter strain.

Selective-agent combinatorial experiments, analyzed by both plating efficiency and monitoring of agar culture GFP-mediated fluorescence assays, resulted in the formulation of the BIN medium described in Table 2. The growth rate of the GFP-expressing EV76 strain on BIN agar was found to be only slightly lower than that on BHIA (Fig. 3). The newly developed BIN selective medium contains cholate salts at a concentration similar to that in CIN agar and contains crystal violet at a concentration similar to those in both CIN and MacConkey agars. However, the level of irgasan is fivefold lower than that in CIN agar, and the formulation is based on the nondefined rich BHIA as the basal medium. The antibacterial agents cefsulodin and novobiocin were omitted, since they were found to be inhibitory to *Y. pestis* at the concentrations routinely used in CIN agar. No attempt was made to include them at reduced concentrations in the final medium, as such a reduction is

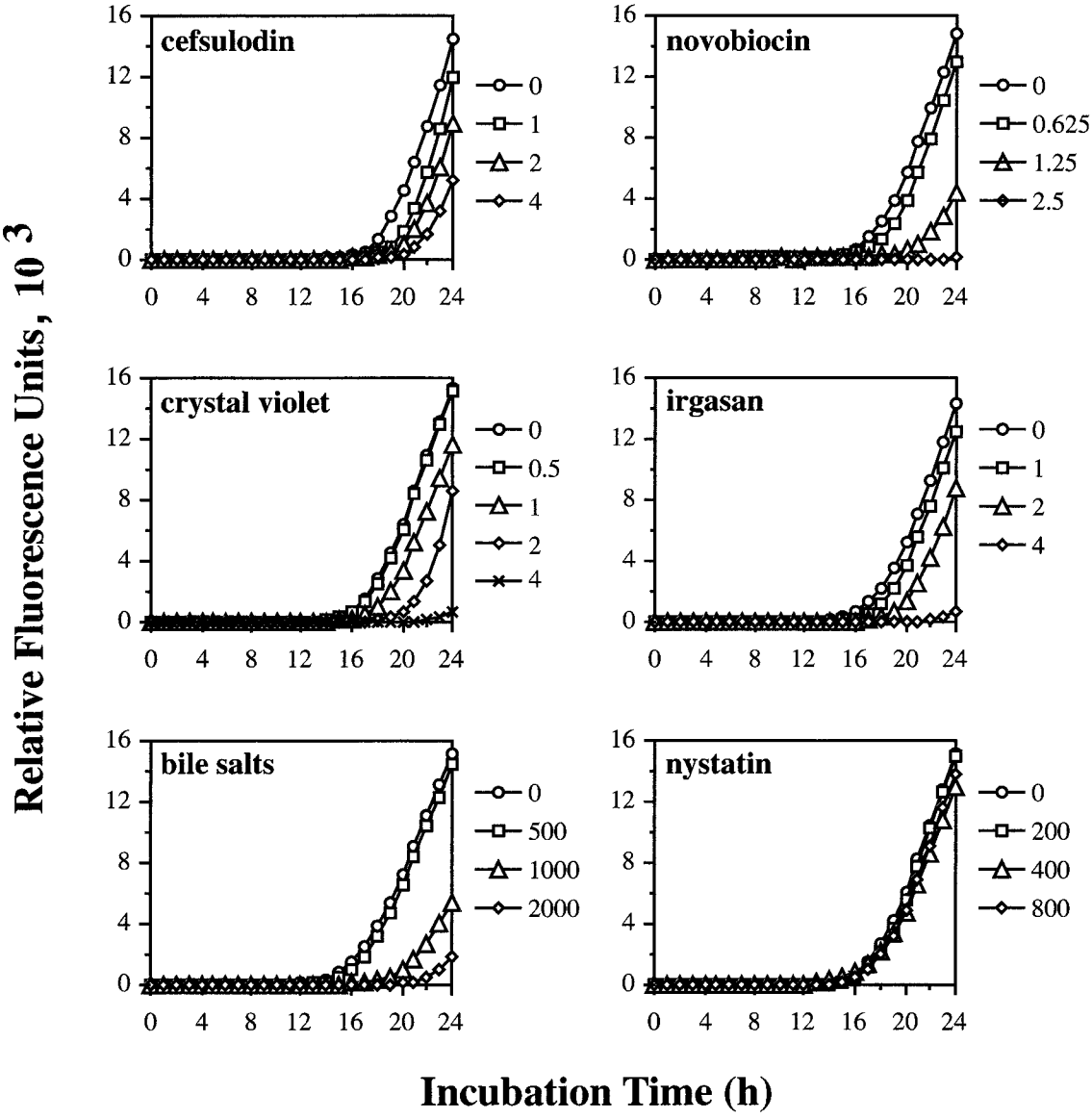

FIG. 2. Fluorescence curves of the GFP-expressing *Y. pestis* EV76 strain on BHIA containing various levels of selective components, incubated at 28°C. The concentrations of cefsulodin, novobiocin, crystal violet, irgasan, and cholate salts (equal amounts of sodium cholate and sodium deoxycholate) are indicated in micrograms per milliliter. The concentration of nystatin is indicated in units per milliliter (USP).

expected to allow growth of some competitor organisms. The mycobacterial agent nystatin has a wide spectrum of antifungal activity, yet it has no activity against bacteria (15). Therefore, it was included in the BIN medium to increase the potential for isolation of *Y. pestis* from environmental samples.

The growth of different *Y. pestis* strains on the BIN medium was compared to that on commercial selective media. All strains were recovered more efficiently on BIN medium than on CIN or MacConkey medium (Table 3). The virulent strain (Kimberley53) had three- to sixfold-higher CFU counts on BIN medium, and the nonvirulent strains exhibited differences in the recoverability on the various media. Generally, BIN medium provided higher recovery values, up to 25-fold over CIN medium and more than 1,000-fold over MacConkey medium, as summarized in Table 3. Our results indicate that *Y. pestis* strains may exhibit variable tolerance toward different selective agents. *Y. pestis* colonies developing on the gray background of BIN medium exhibited a typical light bluish shade in the center and a transparent precipitate in the surrounding area. The bluish-gray center was noticed in colonies of all *Yersinia* strains tested and became more pronounced after further incubation at 4°C.

Selectivity of BIN medium. The lack of antimicrobial agents in the BIN medium could result in reduced selectivity to other competitive organisms. A limited characterization of the BIN selectivity profile was conducted with several gram-positive and gram-negative bacteria which represent both clinically important strains and strains commonly found in the open envi-

TABLE 2. Compositions of *Yersinia* selective media

Chemical agent	Concn (g/liter) in:		
	BIN agar[a]	CIN agar[b]	MacConkey agar[b]
Bacto Proteose Peptone	10[d]	3[c]	3
Calf brains, infusion from	200[d]		
Beef heart, infusion from	250[d]		
Bacto Peptone		17[c]	17
Bacto Yeast Extract		2[c]	
Bacto Agar	15[d]	13.5[c]	13.5
Mannitol		20[c]	
Lactose			10
Dextrose	2[d]		
Neutral red		0.03	0.03
Sodium pyruvate		2[c]	
Magnesium sulfate heptahydrate		0.01[c]	
Disodium phosphate	2.5[d]		
Sodium chloride	5[d]	1[c]	5
Sodium cholate	0.5	0.5	
Sodium deoxycholate	0.5	0.5	
Bacto Bile Salts, no. 3			1.5
Crystal violet	0.001	0.001	0.001
Irgasan	0.0008	0.004	
Cefsulodin		0.004	
Novobiocin		0.0025	
Nystatin	0.025		

[a] Developed by the Israel Institute for Biological Research.
[b] According to Difco.
[c] Component in basal CIN medium.
[d] Component included in the BHIA (Difco), which is added at 52 g/liter. (Growth performance differences between BHIA batches may occur. Batch no. 0418-17-6 provided good growth.)

ronment. BIN medium was found to be completely inhibitory to gram-positive bacteria, yet its selectivity toward gram-negative bacteria was only partial, as summarized in Table 4. In this aspect BIN agar was found to be more selective than MacConkey agar, which allowed the growth of all tested gram-negative strains, but less selective than CIN agar. Nevertheless, all non-*Yersinia* strains tested were easily differentiated from the slow-developing *Y. pestis* colonies, either by growth rate or by morphological characteristics. White *V. cholerae*, yellow-green *P. aeruginosa*, and red-pigmented *S. marcescens* colonies

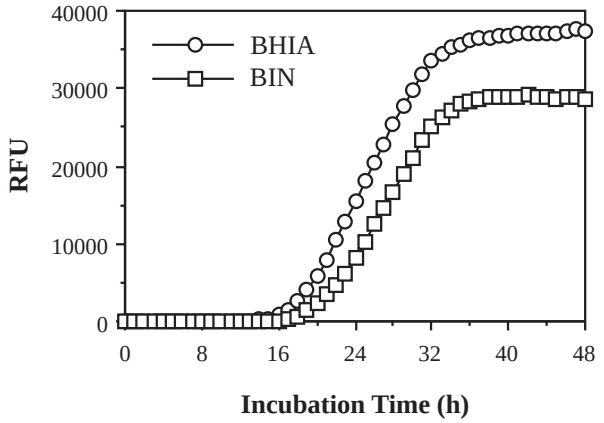

FIG. 3. Growth of *Y. pestis* EV76(pGFPuv) on BIN agar at 28°C. The growth of this strain on BHIA is presented for comparison. RFU, relative fluorescence units.

TABLE 3. Efficiency of colony formation on BIN agar from fresh culture suspensions of different *Yersinia* strains

Species and strain	Efficiency ratio (mean CFU counts ± SD)[a]	
	BIN agar/CIN agar	BIN agar/MacConkey agar
Y. pestis		
Kimberley53	2.9 ± 0.2	5.9 ± 2.7
EV76	25.0 ± 7.1	1.0 ± 0.3
A1122	2.2 ± 0.5	>1,000
TRU	0.8 ± 0.2	>1,000
Y. enterocolitica		
WA	1.4 ± 0.2	0.9 ± 0.1
CDC 497-70	0.9 ± 0.1	1.1 ± 0.1

[a] Aliquots (0.1 ml) from serial 10-fold dilutions ranging from 10^5 to 10 CFU/ml were plated. CFU counts were determined after 48 h.

could be observed already after 1 day, whereas gray *Y. pestis* colonies of ~2 mm in diameter require 2 days to develop. On the other hand, *E. faecalis* growth on BIN agar was very slow, and colony diameters were less then 0.5 mm after 3 days of incubation.

Recovery from dead mice. The effective selectivity of BIN agar together with its ability to support growth of *Y. pestis* at favorable recovery yields prompted us to test its potential advantage in isolation of *Y. pestis* from samples consisting of mixed bacterial population under metabolic stress, such as decomposing carcasses (a model of environmental plague in dead rodents). Twenty mice were inoculated subcutaneously with 100 LD_{50} of the virulent strain *Y. pestis* Kimberley53. After death, decaying carcasses were kept separately at ambient temperature for 3, 5, 8, and 11 days before isolation of *Y. pestis* was attempted. Isolation of *Y. pestis* after 1 day was not attempted, since it has been previously established that $>10^8$

TABLE 4. Selectivities of MacConkey, CIN, and BIN agars against various bacteria

Organism	Growth[a] on:		
	MacConkey agar	CIN agar	BIN agar
Gram negative			
Y. pseudotuberculosis (2 strains)	+	+	+
Y. enterocolitica (2 strains)	+	+	+
S. marcescens	+	+	+[b]
P. aeruginosa	+	±	+[b]
E. faecalis (2 strains)	+	±	+[b]
V. cholerae	+	−	+[b]
S. dysenteriae	+	−	−
S. enterica serovar Typhimurium	+	−	−
E. coli (2 strains)	+	−	−
Gram positive			
S. aureus (2 strains)	−	−	−
B. cereus (2 strains)	−	−	−
B. subtilis	−	−	−
B. thuringiensis	−	−	−
B. amyloliquefaciens	−	−	−
B. anthracis	−	−	−

[a] Growth was monitored for 3 days. +, development of colonies; ±, growth only in heavily inoculated regions; −, no growth.
[b] Colony morphology and growth rate were distinguishable from those of *Y. pestis.*

TABLE 5. Recovery of *Y. pestis* Kimberley53 from spleens of infected dead mice

Day postmortem	Total bacterial CFU per spleen[a]		BIN agar/CIN agar mean ratio (range)
	Y. pestis[b]	Other (10^7)[c]	
3	2×10^6–4×10^7	>30	2.1 (1.4–2.4)
5	5×10^3–4×10^4	>1	4.4 (2.4–6.3)
8	<5–8×10^3	>1	3.8[d]
11	<5	>1	

[a] Spleens from five mice were extracted at each time point (limit of detection, 5 CFU/spleen).
[b] Calculated from recovery on BIN agar and verified by immunofluorescence staining analysis.
[c] Combined from CFU counts on MacConkey agar BHIA, and blood agar.
[d] *Y. pestis* could be recovered from only one mouse at this time point.

Y. pestis bacteria and $>10^{10}$ other contaminating organisms are present at this stage (16). The attempts to isolate *Y. pestis* from samples prepared from spleen homogenates are summarized in Table 5. For all samples, attempts of plating homogenate dilutions or isolation by streaking on rich media (BHIA and blood agar) resulted, after 1 day of incubation, in high CFU counts of fast-growing non-*Yersinia* bacteria, which completely prevented the identification of the slow-developing *Y. pestis* colonies. Similarly, plating on MacConkey agar revealed high numbers of both lactose-positive and lactose-negative colonies, many of which exhibited high growth rates that resulted in coverage of the agar surface long before *Y. pestis* colonies began to appear. The only media that allowed direct and efficient isolation of *Y. pestis* colonies were the selective CIN and BIN media. Background levels of other contaminating organisms on both media were negligible, with CIN agar exhibiting better selectivity but lower recoverability. A continuous decline in *Y. pestis* CFU/spleen (on both CIN and BIN agars) was observed over the period of about 10 days after the animal succumbed, while background bacteria counts remained high at this time as determined by plating on nonselective blood agar and BHIA or on MacConkey agar. As long as *Y. pestis* counts were above $\sim 10^6$ CFU/spleen, the recovery on BIN agar was about twofold higher than that on CIN agar. However, as *Y. pestis* counts in the decomposing tissues declined (probably due to severe metabolic stress or competition conditions), the advantage of BIN agar became more pronounced, as the BIN/CIN ratio increased to about fourfold (Table 5). Similar results were obtained with samples taken from livers of the same mice. These results clearly emphasize the advantageous growth-supportive characteristic of BIN agar. Furthermore, similar results were obtained with *Y. enterocolitica* bacteria recovered from dead mice following oral infection with lethal doses of *Y. enterocolitica*. The recoverability of *Y. enterocolitica* from spleens of dead mice 5 days postmortem was three- to sixfold higher on BIN agar than on CIN agar (data not shown), indicating the advantage of BIN agar for isolation of probably all clinically important *Yersinia* strains from an environment imposing metabolic stress.

In summary, the BIN medium is superior to the WHO-recommended selective medium, MacConkey medium, as well as to the commercial CIN medium for isolation and recovery of *Y. pestis* from pure and fresh samples as well as from background environments where the bacterium is expected to be under stress.

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