APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1995, p. 3379–3384 Vol. 61, No. 9
0099-2240/95/$04.00+0

Oxidative Metabolism in Nonculturable *Helicobacter pylori* and *Vibrio vulnificus* Cells Studied by Substrate-Enhanced Tetrazolium Reduction and Digital Image Processing

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Received 17 April 1995/Accepted 30 June 1995

Growing and nonculturable cells of *Helicobacter pylori* and *Vibrio vulnificus* were studied for the capacity to reduce tetrazolium salts in order to elucidate the possible physiological basis for the proposed "viable but nonculturable" (VNC) state. Initial difficulties in obtaining consistent reduction of ρ-iodonitrotetrazolium violet (INT) by *H. pylori* led us to develop a method for studying the effect of adding exogenous substrates on these reactions. The established procedure provided a profile of substrate enhancement of oxidative activity revealed by INT reduction which was related to both the identity and physiological state of the organism studied. Representation and interpretation of these enhancement profiles were facilitated by digital image processing. Nonculturable cells of *H. pylori* produced by carbon and nitrogen starvation in air lost all INT-reducing capacity in 24 h when stored at 37°C, while 99% of those produced at 4°C retained oxidative activity for at least 250 days when tested in the presence but not in the absence of succinate, α-ketoglutarate, or aspartate. Activity was detected at similar levels in cells with coccoid and spiral shapes. In contrast, only 1% of nonculturable cells of *V. vulnificus*, produced under conditions previously reported to induce the VNC state in this organism, retained intrinsic INT-reducing capacity; no substrate-enhanced activity occurred in the remainder of the population. Thus, there was no common pattern of oxidative activity indicative of a VNC state in both test organisms. Nonculturable cells of *H. pylori* can retain several different oxidative enzyme activities; whether these indicate viability or the persistence of cells as "bags of enzymes" remains to be established.

Helicobacter pylori was first isolated in 1982 (13) and has now been shown to cause gastritis, is implicated in peptic ulcer formation, and may also be a contributory factor in gastric carcinoma (6). Although more than half the world's population have been infected with this organism, its reservoirs and mode of transmission have yet to be established. In particular, the failure to repeatedly detect the organism in the environment by culture despite epidemiological evidence that waterborne and fecal-oral transmission occur (9, 11) has led to the suggestion that *H. pylori* is capable of transition to a "viable but nonculturable" (VNC) state (2, 17, 22). If confirmed, this capacity, which has been associated with morphological transformation from spiral to coccoid cells (4), may also explain why initial clearance of the organism from the stomach by antimicrobial therapy is often followed by recurrence (5).

The putative VNC state has been considered to represent a specific form of dormancy occurring in nonsporulating organisms (17). Cells in this state cannot be grown by the culture method normally used for the organism concerned but are believed to have the capacity to return to the vegetative state when exposed to appropriate stimuli. Although nonculturable cells can readily be produced in laboratory microcosms and detected in environmental samples, the means by which they can be determined to be viable are not well established. Two lines of approach have been used, the detection of some form of activity either at the single-cell or whole-culture level and the eventual recovery of vegetative cells in conventional culture (14). In the former case the relationship between the activity detected and viability is uncertain, while the latter has been reported only on a few occasions (2). While the long-term aim should be to correlate the two phenomena, both approaches have yielded evidence for VNC forms of the organism which has perhaps been most extensively studied in this context, *Vibrio vulnificus* (14, 18, 28).

Tetrazolium salts have been used extensively as cytochemical indicators of oxidative metabolism in bacteria. In particular, ρ-iodonitrophenyl tetrazolium violet (INT) (20, 29) and cyanoditolyl tetrazolium chloride (CTC) (19, 21) have been advanced as indicators of viability. The factors affecting the outcome of reactions involving these reagents have been investigated in this laboratory as part of a program aimed at developing well-standardized means of determining the physiological state of individual bacterial cells by light microscopy (24). In the present study we have applied established and newly developed tetrazolium reaction procedures to determine the phenotype of nonculturable cells of *H. pylori* formed under physiologically well-defined conditions. Tetrazolium reduction in *H. pylori* has been compared with that observed with nonculturable cells of *V. vulnificus* produced under conditions previously reported to stimulate formation of VNC cells by this organism (14). The results, which were assessed with the assistance of digital image processing, clearly demonstrate retained capacity for tetrazolium salt reduction in nonculturable cells of *H. pylori* and that nonculturable cells produced under different conditions have different phenotypes.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. *H. pylori* CP END3 (25), *Escherichia coli* (NCTC 10499), and *V. vulnificus* C7184T (23) were used throughout the study. *H. pylori* and *E. coli* were grown at 37°C, respectively, on Columbia agar

base (Becton Dickinson, Cockeysville, Md.) supplemented with 5% defibrinated horse blood under microaerobic conditions (Campypak; Becton Dickinson) and aerobically on nutrient agar plates (Lab M; Amersham, Bury, England). Cultures of *V. vulnificus* were grown and maintained on VNSS agar (16) as described by Weichart and colleagues (27). Surface culturable counts (SCC) of *H. pylori* and *V. vulnificus* were made on 5% blood agar and VNSS agar, respectively.

Carbon-nitrogen (C-N) starvation. *H. pylori* cells were harvested from edges of colonies grown for 22 h on agar directly into sterile phosphate-buffered saline (PBS) at pH 7.2, while *V. vulnificus* cells were grown to mid-exponential phase at 30°C in VNSS broth. The cells were washed three times and resuspended in PBS (*H. pylori*) or nine-salt solution (*V. vulnificus*) (16) to a density of $4 \times 10^7 \pm 2 \times 10^7$ CFU ml^{-1}. These preparations were divided into three 15-ml microcosms in screw-cap glass universal bottles (20 ml) which were maintained at 37°C, room temperature (15 to 25°C), and 4°C for *H. pylori* and at 30°C, room temperature, and 4°C for *V. vulnificus*. At the initiation of and at weekly intervals during starvation, the microcosms were mixed well by vortexing and a 1-ml sample was taken for assessment of culturability, microscope-based counts, and tetrazolium reactions.

SCC were determined by the drop plate method (7). Serial 10-fold dilutions were made in PBS or nine-salt solution, and 20-μl aliquots were dropped onto the appropriate medium in triplicate. When the culturability decreased, the total sample volume was increased to 160 μl to give a minimum culturable-cell detection limit of 6.25 CFU ml^{-1}. Plates were incubated for 24 h (*V. vulnificus*) and 48 h (*H. pylori*), colony counts were determined, and then the plates were reincubated for a further 24 h and the counts were rechecked. No increases were observed on reincubation.

Microscope-based counts. Total cell counts and cell morphology assessments were made by immobilizing cells onto aminopropylsilane-coated coverslips as previously described (1). The resulting monolayers were fixed for 30 s with freshly prepared 4% PBS-buffered paraformaldehyde, rinsed in distilled water, gently blotted, and allowed to air dry before being mounted on the coverslips in PBS and sealed with clear nail varnish. Total cell counts and the number of cells of each shape were determined by phase-contrast microscopy. The shapes recognized were spiral, intermediate (V, U, and horseshoe-shaped cell outlines), and coccoid (8). Counts were made on complete fields of view, and at least 300 cells were assessed per monolayer. Results were recorded by video microscopy (Panasonic WCVL 700 camera) onto Fuji SVHS Pro videotapes with a Panasonic FS200 video recorder.

Tetrazolium reactions. All reactions were carried out in sterile 1.5-ml microcentrifuge tubes. Samples (50 μl) of the cells to be tested were incubated with 50 μl of 10 mM INT or 4 mM CTC plus 10 μl of PBS or 10 μl of one of the following substrates (1 M in PBS): succinate, pyruvate, glucose, glutamate, aspartate, α-ketoglutarate, malate, and citrate. The tubes were incubated for 2 h at 37°C for *H. pylori* and *E. coli* and at 30°C for *V. vulnificus*, after which the cells were immobilized, fixed, and mounted and their microscopic appearances were recorded as described above. CTC reactions were observed by fluorescence microscopy (19). A fresh overnight culture of *E. coli* was tested on each occasion as a positive reaction control. The proportion of tetrazolium-reducing cells was determined by comparing the phase-contrast and bright-field (Kohler illumination) images; the former enables detection of all cells present, while the latter shows only those cells containing formazan deposits. A blank image with no cells was recorded for each slide.

Image processing. Recorded images were processed with a DT2867LC frame grabber and PC_Image (Foster-Findlay & Associates, Newcastle upon Tyne, United Kingdom) installed in a 33-MHz 486 IBM-compatible microcomputer. Analog video signals were digitized and stored at an 8-bit resolution (256 distinct grey levels where 0 = darkest or most opaque objects and 255 = 100% transmission for bright-field illumination). For comparative displays the images were shade corrected by using the blank image to compensate for uneven illumination and were intensity inverted in order to show the formazan deposits as white against a dark background. This provided images which were strictly comparable by objective criteria. For quantitative analyses the total amount of INT-formazan per cell (arbitrary units) was assessed to confirm the validity and estimate the relative levels of activity represented by the subjective grading used in Table 1. Microspectrophotometry of formazan deposits has been used extensively in quantitative cytochemistry to measure site-specific enzyme activity (26), and a related approach has been used here. To do this, phase-contrast and shade-corrected bright-field images were displayed in parallel. A binary mask was made to overlay the formazan deposits in the bright-field image. The optical weight, an estimate of the integrated optical density of all the deposits within a cell identified by the phase image, was then determined and averaged over 10 well-separated cells which were subjectively considered to be representative of the population as a whole (optical weight = $\Sigma_1^n[\log 255.5 - \log (gi + 0.5)]$, where n is the number of pixels representing the formazan deposits within a cell and gi is the intensity value of the ith pixel in the series 1 to n).

RESULTS

Initial experiments established the overall pattern of decline in SCC and development of coccoid forms of *H. pylori* in response to C-N starvation. Consistent SCC, shapes, and tetrazolium results were obtained when inocula were prepared from washed-cell suspensions derived from 22-h agar cultures. Older cultures had high initial proportions of coccoid and intermediate-shaped cells (20% at 72 h) and could not be used to develop the tetrazolium assays because of substantial variation in both the proportion of formazan-positive cells (5 to 60%) and the amount of deposit per cell. Maintenance of temperature control was found to be an important factor in the tetrazolium reactions, but provision of a microaerobic atmosphere produced no discernible enhancement over aerobic incubations. INT was preferred to CTC because it was more reactive (higher proportion of positive cells and larger deposits) and gave less extracellular formazan.

TABLE 1. Effects of addition of exogenous substrate on INT reduction in growing cells of *E. coli*, *H. pylori*, and *V. vulnificus*

Organism	Effect of addition of substrate[a]							
	GLC	GLT	AKG	ASP	MAL	PYR	CIT	SUC
E. coli	+	+	+	++	+	++	−	+++
H. pylori	0	+	++	+	++	++	+	+++
V. vulnificus	+++	+	+	+	+	++	++	+

[a] GLC, glucose; GLT, glutamate; AKG, α-ketoglutarate; ASP, aspartate; MAL, malate; PYR, pyruvate; CIT, citrate; SUC, succinate. The substrate effects on INT reduction were graded in comparison with endogenous reduction. −, suppression; 0, no additional reduction; +, ++, and +++, 2 to 3, 4 to 7, and 10 to 20 times, respectively, the level of reduction per cell as determined by image analysis (see text).

Substrate-enhanced tetrazolium reactions. INT reactions were further studied by examining the potential of exogenous substrates to enhance reduction in growing cells. Results obtained with *H. pylori*, *E. coli*, and *V. vulnificus* are shown in Fig. 1. The application of a range of substrates can be seen to provide a profile of different reaction intensities which distinguish between the phenotypes of the organisms at the cellular level. The extent of tetrazolium reduction in an individual cell can be recognized from the intensity, size, and number of formazan deposits. A comparative summary based on direct observation of the reactions demonstrated in Fig. 1 is shown in Table 1. (Note that more fields of view than can be shown in Fig. 1 were used to make the assessments in Table 1.) While quantitative assessment is possible in terms of an estimate of total formazan per cell, it is beyond the scope of this report. However, a small-scale analysis based on 10 representative cells from each of the substrate-organism combinations was used to estimate the quantitative differences represented by the subjective grading system used in Table 1 and to confirm the categorization of reactions. The organisms clearly differ in the patterns of their responses to the eight substrates; the strongest enhancement was observed with succinate for *E. coli* and *H. pylori* and glucose for *V. vulnificus*. It should be noted that in the optimized 22-h preparation of *H. pylori*, a significant and consistent level of endogenous-INT reduction was obtained. In light of these results we elected to study all samples from starved populations of *H. pylori* and *V. vulnificus* with INT in the absence and presence of succinate (INT and INT-S assays, respectively). Selected samples were also studied with all eight substrates to determine whether new patterns of substrate stimulation might develop during the course of starvation.

Effects of starvation at 37°C, room temperature, and 4°C. When cells of *H. pylori* were prepared from 22-h agar cultures as described above and subjected to C-N starvation at three different temperatures, SCC declined rapidly to below the limit of detection (6.25 CFU ml^{-1}) in microcosms maintained at

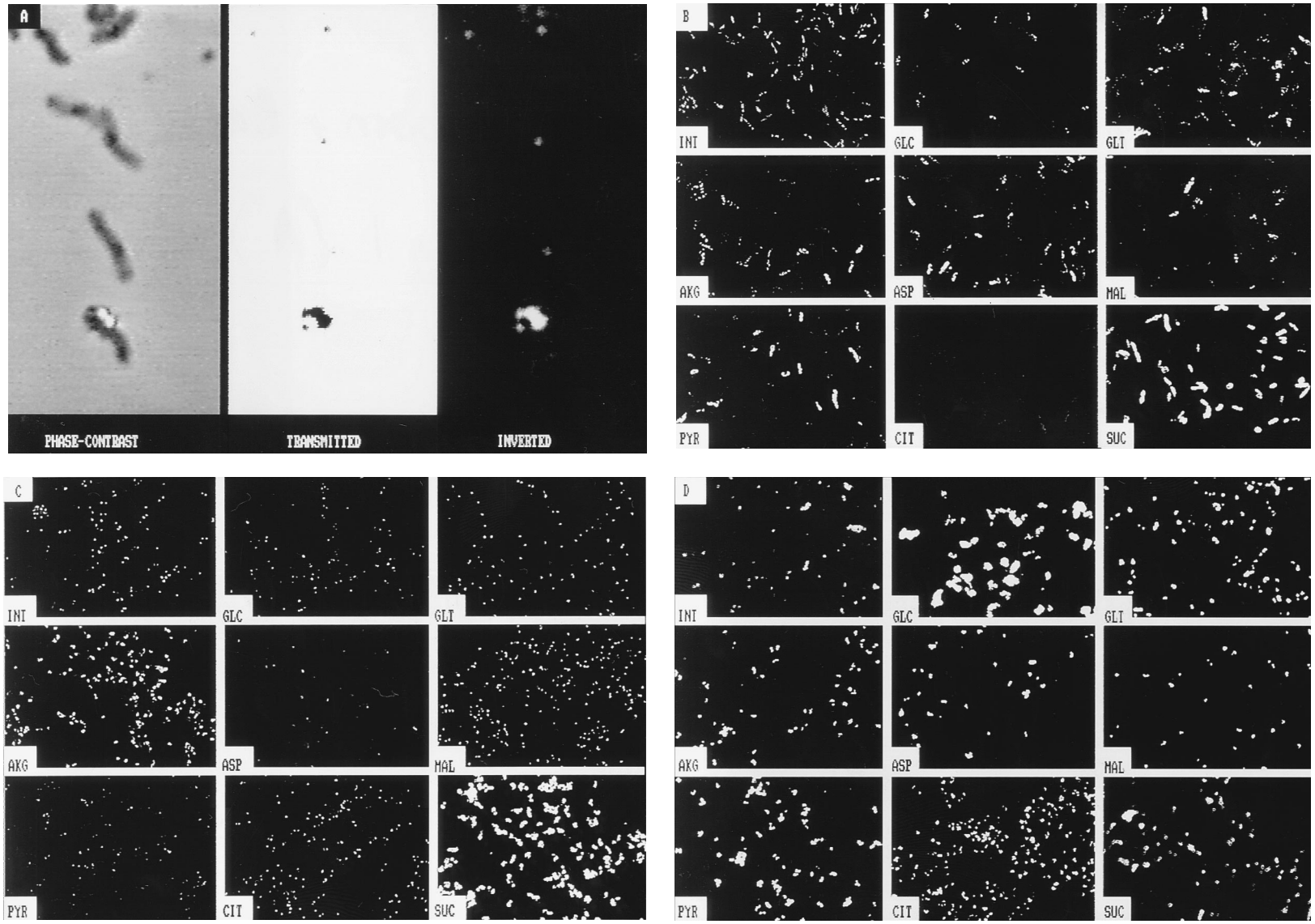

FIG. 1. Substrate-stimulated tetrazolium reactions in growing cells of *E. coli*, *H. pylori*, and *V. vulnificus*. (A) Corresponding phase-contrast, transmitted (bright-field), and intensity-inverted bright-field images (8 by 16 μm) of *H. pylori* showing INT reduction without substrate addition. The phase-contrast image shows the cell-localized formazan and the overall cell density, while the bright-field image shows formazan deposition without the cell outlines. Intensity-inverted images have been used to optimize the display of weak formazan deposits. The white spots are the exact inverse of the dark spots on the adjacent bright-field image and represent individual cell-localized formazan deposits, the intensity and size of which are proportional to the local oxidative activity. (B to D) Intensity-inverted bright-field images showing substrate-stimulated formazan deposition in *E. coli* (B), *H. pylori* (C), and *V. vulnificus* (D). Each panel comprises nine images demonstrating the pattern of INT reduction occurring in the presence of different exogenous energy sources. GLC, glucose; GLT, glutamate; AKG, α-ketoglutarate; ASP, aspartate; MAL, malate; PYR, pyruvate; CIT, citrate; SUC, succinate. The number of cells per unit area was within ±25% for all the images shown; the intensities and sizes of deposits are the main indicators of oxidative activity, but, within the limits indicated, the relative frequencies of reduction-positive cells are also reflected. Each image (B to D) represents a microscopic field of 48 by 32 μm.

TABLE 2. Effect of C-N starvation at three temperatures on culturability, shape, and INT-reducing activity of *H. pylori*

Time (days)	Temp	% Total cell count			
		SCC	Coccoid	INT[a]	INT-S[a]
0	RT[b]	100	3	85	100
7	37°C	$<2 \times 10^{-5}$	23	<0.1	<0.1
	RT	$<2 \times 10^{-5}$	14	<0.1	99
	4°C	10^{-3}	11	<0.1	99
14	37°C	$<2 \times 10^{-5}$	32	<0.1	<0.1
	RT	$<2 \times 10^{-5}$	17	<0.1	99
	4°C	2×10^{-5}	13	<0.1	99

[a] Percent cells with detectable formazan deposit. INT-S, succinate enhanced.
[b] RT, room temperature.

TABLE 3. Effect of C-N starvation at three temperatures on culturability, shape, and INT-reducing activity of *V. vulnificus*

Time (days)	Temp	% Total cell count			
		SCC	Coccoid	INT[a]	INT-S[a]
0	RT[b]	100	<0.1	66	76
21	30°C	5	<0.1	3	2
	RT	20	<0.1	23	27
	4°C	6	23	9	6
49	30°C	0.3	<0.1	1	1
	RT	2	<0.1	4	5
	4°C	2×10^{-5}	38	1	1

[a] Percent cells with detectable formazan deposit. INT-S, succinate enhanced.
[b] RT, room temperature.

37°C and room temperature (1 and 3 days, respectively). The 4°C microcosm retained culturability at 7 days, but SCC fell below the limit of detection at 14 days. The total cell counts at each temperature remained within 15% of their initial values throughout the experiment (49 days). The proportion of cells capable of reducing INT without added substrate also declined rapidly to less than 0.1% of the total cell count within 7 days of initiation of starvation at all three temperatures. In contrast, the majority of nonculturable cells obtained in the room temperature and 4°C microcosms retained the capacity to reduce INT in the presence of succinate. Together with estimates of the proportion of coccoid cells, these results are summarized in Table 2 for samples taken at 7 and 14 days after starvation was initiated. Although the weekly sampling program was continued through 49 days, there was no significant change in any of the measured indices apart from a gradual increase in the proportion of coccoid cells. The succinate-enhanced formazan deposits in nonculturable cells obtained at both 4°C and room temperature filled the entire cell, irrespective of its shape (coccoid, intermediate, or spiral); deposits in cells from the room temperature microcosm were less intense than their 4°C counterparts. In samples taken at 95 and 250 days, only the cells maintained at 4°C retained succinate-stimulated INT-reducing activity. The proportion of coccoid cells had increased to >90% in these later samples.

In order to compare our results with a well-characterized system in which nonculturable cells have been studied in detail, we determined the INT reactivity and SCC in samples from microcosms inoculated with *V. vulnificus* and maintained at 30°C, room temperature, and 4°C. The results are summarized in Table 3. Colony counts fell below the limit of detection after 49 days at 4°C, while culturability was sustained at the other temperatures. In contrast to *H. pylori*, growing *V. vulnificus* cells have a high level of intrinsic INT-reducing activity which is only moderately stimulated by succinate. This level of stimulation was not detectable in populations undergoing starvation in which the INT and INT-S counts were essentially the same. The pattern of change in INT reactivity elicited by starvation or temperature stress was also very different in *V. vulnificus*. Formazan positivity fell to between 1 and 5% of the total cell count at 49 days irrespective of the microcosm temperature and was apparently independent of the culturability, which had fallen 10^7-fold in one case (4°C) and only 50-fold in another (room temperature).

A comparison of the morphological changes which occurred in the two organisms revealed that *H. pylori* showed the highest proportion of transformation to coccoid cells at 37°C and the least at 4°C, whereas *V. vulnificus* showed morphological changes only at 4°C (Tables 2 and 3).

Nonculturable cells of both organisms were also studied with the full range of substrates established above. The objective was to determine which activities were sustained and whether any new patterns might develop in response to starvation. *H. pylori* showed well-preserved α-ketoglutarate- and succinate- and some aspartate-related activity (Fig. 2). The relative preservation of aspartate reactivity is particularly noteworthy because this substrate gave only a minor enhancement of formazan deposition in fresh cells, while reduction stimulated by several other substrates (e.g., glutamate) was lost. In contrast, formazan deposition occurred at a low frequency (1 in 10^2 to 10^3 cells) in nonculturable populations of *V. vulnificus* and substrate addition did not raise the proportion of formazan-positive cells (data not shown). In view of this low frequency, it was not possible to determine whether the pattern of substrate stimulation had changed.

DISCUSSION

Initial difficulties in obtaining reproducible INT results with culturable preparations of *H. pylori* stimulated us to study the effects of adding oxidizable substrates to the incubation medium. Application of the developed method to 22-h cell preparations and comparison of the results with those obtained with *E. coli* and *V. vulnificus* led to the recognition that the patterns of formazan deposition obtained constituted phenotypic descriptions at the single-cell level which differed according to the identities of the cells and their physiological states. It was hoped, therefore, that tetrazolium reactions obtained with potentially heterogeneous nonculturable populations (e.g., viable and nonviable cells) might provide insights into the biochemical profiles of the cells that composed them. It must be emphasized that while tetrazolium salts are generally used by histochemists to detect and measure specific oxidoreductase and other enzyme activities, in the present context INT detects the overall rate of production of reducing equivalents within cells and the effect of adding substrate on that rate.

The tetrazolium studies showed that the temperature at which nonculturable cells of *H. pylori* are formed influences their phenotype. Loss of culturability at 37°C resulted in >99% of cells with no detectable reducing activity, while at 4°C substrate-stimulated INT formazan deposition was readily demonstrable in most cells. In contrast, tetrazolium results could be clearly attributed only to nonculturable *V. vulnificus* cells formed at 4°C; thus, it was not possible to determine whether different phenotypes were produced at different temperatures.

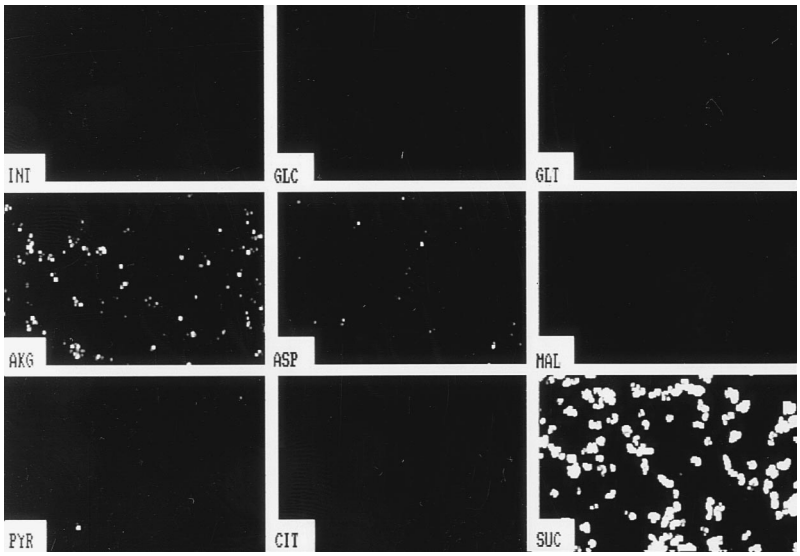

FIG. 2. Substrate-stimulated tetrazolium reactions in nonculturable cells of *H. pylori*. Intensity-inverted images were processed as described for Fig. 1.

The formazan deposits obtained in nonculturable *H. pylori* cells were not particularly associated with coccoid cells. Although this appears to contradict the view that coccoid and VNC cells are synonymous, it was observed that nonculturable spiral populations eventually become >90% coccoid.

The substrate-enhanced formazan deposition obtained in nonculturable cells of *H. pylori* showed retained activity with α-ketoglutarate, aspartate, and succinate. Relative to the pattern observed in growing cells, there was a small increase in aspartate-stimulated reduction but otherwise the results indicated a general decline in activity. While production of reducing equivalents from the membrane-bound succinate dehydrogenase complex is mediated by flavoproteins, α-ketoglutarate oxidation implies the presence of both NAD^+ and coenzyme A. Aspartate-mediated formazan deposition could result either from transamination to α-ketoglutarate or from an amino acid oxidase; in the former case significant levels of α-ketoglutarate, NAD^+, and glutamate dehydrogenase would be required, while in the latter, flavin adenine dinucleotide appears to be necessary (10). Since there was evidently too little α-ketoglutarate retained to provide for formazan deposition in the absence of exogenously added substrate and glutamate addition showed no stimulatory activity, we favor the amino acid oxidase pathway to explain the observed reduction. The retained stimulation of INT reduction by α-ketoglutarate and aspartate therefore indicates that significant concentrations of pyridine nucleotides were present and suggests a higher level of metabolic integrity than the simple retention of selected enzymes.

Our findings are in general agreement with previous studies of nonculturable *H. pylori* cells in which activities were demonstrable only in preparations maintained at 4°C. Both autoradiographic (22) and 5-bromodeoxyuridine incorporation (3) studies indicated retained enzyme activity at this temperature.

The relationship between the nonculturable INT-reducing *H. pylori* cells observed and putative VNC cells is uncertain. In particular, the results with *V. vulnificus* did not assist in identifying a phenotype which could be clearly assigned to VNC cells; INT reduction was at such a low frequency ($<10^{-2}$) in nonculturable populations of this organism that it was impractical to determine whether the pattern of reactivity had changed from that observed in growing cells. This low frequency was also observed by Nilsson and colleagues, and they suggested that INT underestimates the proportion of viable cells in nonculturable populations of *V. vulnificus* (14), although later studies cast doubt on this interpretation (27).

Positive tetrazolium reactions indicate retained enzyme activity which can be used as a means of characterizing the phenotype of nonculturable cells. INT reduction in the absence of an exogenous substrate indicates the presence of an endogenous substrate(s) for oxidative reactions, and some substrate-stimulated reactions indicate the presence of multiple enzymes and the potential to form activated carrier molecules such as NADH and NADPH. This level of metabolic integrity is not inextricably linked to viability. Indeed, we have observed implicitly higher orders of integrity in the form of retained capacity to respond to an enzyme induction stimulus with de novo synthesis of β-galactosidase in nonculturable cells of *Salmonella enteritidis* and *E. coli* (12, 15). These results showed that genetic regulatory functions can be intact in nonculturable cells even though the viability of the cells involved was no more certain than that of the *H. pylori* cells studied here.

We conclude that nonculturable *H. pylori* cells generated at different temperatures show different phenotypes and that significant levels of oxidoreductase activities are retained at 4°C. No clear relationship between the phenotypes of nonculturable cells of *H. pylori* and putatively VNC cells of *V. vulnificus* was identified. However, the substrate-stimulated INT reduction procedure developed provides new opportunities to characterize the phenotypes of nonculturable cells derived from bacterial isolates propagated in vitro and cells of organisms which are as yet unculturable.

ACKNOWLEDGMENTS

We thank S. Kjelleberg, J. Oliver, and D. Weichart for helpful discussions.

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