INFECTION AND IMMUNITY, Feb. 2004, p. 1192–1194 Vol. 72, No. 2
0019-9567/04/$08.00+0 DOI: 10.1128/IAI.72.2.1192–1194.2004

Hydrogen Peroxide-Mediated Killing of *Caenorhabditis elegans*: a Common Feature of Different Streptococcal Species

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Received 6 October 2003/Returned for modification 7 November 2003/Accepted 10 November 2003

Recently, we reported that *Streptococcus pyogenes* kills *Caenorhabditis elegans* by the use of hydrogen peroxide (H_2O_2). Here we show that diverse streptococcal species cause death of *C. elegans* larvae in proportion to the level of H_2O_2 produced. H_2O_2 may mask the effects of other pathogenicity factors of catalase-negative bacteria in the *C. elegans* infection model.

Streptococcal species other than group A streptococci (GAS; *Streptococcus pyogenes*) can cause a wide range of diseases in animals and humans, including nonsymptomatic commensal-like carriage, skin infections, septicemia, arthritis, endocarditis, otitis media, pneumonia, and meningitis (1, 5, 11). A frequent cause of severe diseases is *S. pneumoniae* (pneumococcus) (1, 11), while closely related viridans streptococci (*S. mitis* and *S. oralis*) are commensals of the human oral cavity (12). Group B streptococci (GBS; *S. agalactiae*) predominantly infect neonates (15, 16). Among the group C streptococci (GCS) and group G streptococci (GGS) are mainly animal pathogens (e.g., *S. zooepidemicus* and *S. canis*) (5). To gain further insight into the complex streptococcus-host interactions, additional animal models are needed. *Caenorhabditis elegans* has been introduced as a new, facile, cheap, and genetically amenable model organism for the study of the pathogenesis of several human pathogens (reviewed in reference 6). The first streptococcal species shown to kill *C. elegans* was *S. pneumoniae*, although the mechanism of killing remained unclear (8). Recently, we described that *S. pyogenes* kills *C. elegans* within 24 h and that this killing is exclusively mediated by H_2O_2 (10). Since H_2O_2 production under aerobic conditions might be a common feature of the catalase-negative streptococci, we considered H_2O_2 toxicity to be the general characteristic by which streptococci kill *C. elegans*.

To test this hypothesis, killing assays were performed using *C. elegans* N2 (Bristol). Fifteen GBS strains (6 serotypes), 13 GCS (7 *S. zooepidemicus*, 3 *S. dysgalactiae*, 2 *S. equisimilis*, and 1 *S. equi*) strains, 19 GGS strains, 2 viridans streptococcus strains (*S. mitis* and *S. oralis*), and 15 pneumococcus strains (10 serotypes) were used in this study. GAS strains SP1 and SP2 (14) and *Escherichia coli* OP50 (3) were used as control strains.

Killing assays were performed as described previously (10). Briefly, for the liquid killing assay, sterile *C. elegans* L1 larvae were added to bacterial logarithmic-phase cultures in fresh diluted Todd-Hewitt broth supplemented with 0.5% yeast extract (THY), and viability at 25°C was scored. H_2O_2 production in the liquid killing assay was measured photometrically by using horseradish peroxidase and phenol red as substrate (10). Catalase at a final concentration of 2,000 U/ml was added to control cultures prior to the addition of *C. elegans* larvae to eliminate H_2O_2.

For the solid killing assay, GAS, GBS, GCS, GGS, and viridans streptococci were grown overnight at 37°C in THY, while *S. pneumoniae* cultures were grown for only 5 h in brain heart infusion (BHI) medium. BHI medium was used instead of THY in order to compare our results with those published earlier by Garsin et al. (8). The bacterial overnight cultures were plated (100 μl) on nematode growth medium agar and incubated aerobically at room temperature for 24 h. For *S. pneumoniae*, 100 μl of culture was plated on BHI agar and incubated at 37°C in a 5% CO_2–95% air atmosphere for 24 h according to the protocol of Garsin et al. (8). Incubation with *C. elegans* nematodes at 25°C under aerobic conditions and scoring of viability were performed as previously described (10).

Killing capacity and H_2O_2 production in the liquid assay were tested over a time course of 24 h for three representative strains of GBS, GCS, GGS, and *S. pneumoniae*, as well as for two strains of viridans streptococci (Table 1). In addition, two GAS strains were used as controls: an H_2O_2-negative (SP1) and an H_2O_2-positive (SP2) strain (14). *E. coli* OP50 was used as a negative control. None of the GBS strains were lethal to *C. elegans*, while one of three GCS strains, two of three GGS strains, and all three *S. pneumoniae* strains killed the nematode worms. The control strains GAS SP1 and *E. coli* OP50 did not influence the viability of *C. elegans*, whereas GAS SP2 was lethal. There was a positive correlation between H_2O_2 production by the bacteria and killing of the nematodes (Table 1). For all strains, killing of *C. elegans* was preceded by accumulation of H_2O_2. All strains accumulated maximum H_2O_2 concentrations after 6 h, and killing plateaus were reached after 24 h. Figure 1 shows H_2O_2 production and killing curves within 24 h for a representative strain. Catalase prevented killing of *C. elegans* by all strains in both the liquid assay (Table 1) and the solid assay (data not shown). The killing capacity of all strains significantly correlated with the amount of H_2O_2 produced ($r^2 = 0.99$, $n = 15$, $P < 0.0001$; GraphPad Prism linear-regression analysis).

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TABLE 1. Killing of *C. elegans* by different streptococcal species is proportional to the level of H_2O_2 production[a]

Group and/or species	Strain	H_2O_2 concn (mM)	% Killed	
			Without catalase	With catalase[b]
GAS				
S. pyogenes	SP1	0.051	<1	<1
	SP2	1.500	100	<1
GBS				
S. agalactiae	0171	0.007	<1	0
	0176	0.007	0	0
	MM96000115	0.007	<1	<1
GCS				
S. dysgalactiae	Dys 97/21	1.600	100	<1
S. equi	Equi 90 43569	0.029	<1	0
S. equi subsp. *zooepidemicus*	Zoo 82 43572	0.110	<1	0
			<1	0
GGS	Kiel 13	0.047	<1	0
	G22	1.500	100	0
	G25	1.400	100	0
S. pneumoniae	ATCC 6303	1.700	100	0
	ATCC 6319	1.500	100	<1
	ATCC 10368	1.700	100	<1
S. mitis	ATCC 49456	1.610	100	0
S. oralis	ATCC 35037	1.520	100	<1
E. coli	OP50	0.061	<1	0

[a] Data indicate H_2O_2 production after 6 h and percent killing after 24 h in the liquid assay (means, $n = 2$). H_2O_2-producing (SP2) and -nonproducing (SP1) GAS strains (14) and *E. coli* OP50 were used as controls.
[b] Catalase was nontoxic for streptococci.

To find out whether a majority of strains in each group had the potential to kill, the number of strains tested for killing efficacy was increased. A total of 15 GBS, 13 GCS, 19 GGS, 2 viridans streptococcus, and 15 pneumococcus strains were evaluated for their capacity to kill *C. elegans*. None of the GBS strains killed *C. elegans*, whereas 4 of 13 (31%) of the GCS strains, 11 of 19 (58%) GGS strains, and 15 of 15 (100%) pneumococcus strains, as well as both oral streptococcus strains (100%), killed *C. elegans* in the liquid assay (Table 1).

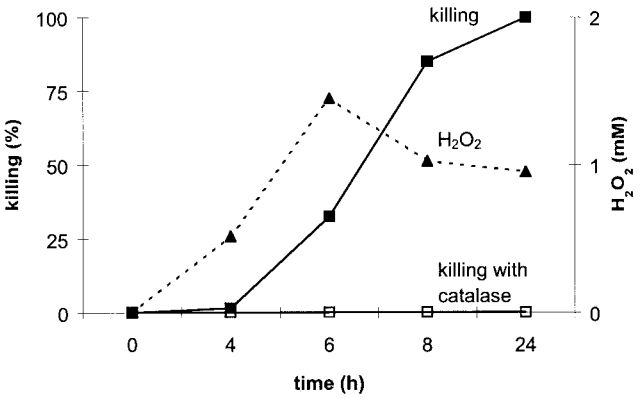

FIG. 1. H_2O_2 production and nematode killing by *S. pneumoniae* strain ATCC 6319. Results are mean values from two representative experiments. H_2O_2 production is indicated by triangular symbols, nematode killing without catalase is shown with closed square symbols, and killing with catalase is depicted with open square symbols.

As we observed previously (10), killing was less pronounced in the solid assay. Strains that were nonlethal in the liquid assay also did not kill on solid medium. None of the GBS, GCS, GGS, or viridans streptococcus strains killed the nematodes after 24 h on solid plate medium, whereas 12 of 15 (80%) pneumococcus strains were lethal.

We show here that besides GAS, other streptococci could kill *C. elegans*. As was reported previously for GAS (10), we found here that pneumococci, viridans streptococci, some GCS, and GGS produced similar amounts of H_2O_2 and showed similar killing kinetics as equimolar amounts of pure H_2O_2. Killing by all strains depended completely upon the amount of H_2O_2 produced and could be prevented with catalase. None of the GBS produced H_2O_2; therefore, they did not affect the viability of *C. elegans*.

There was no correlation between human pathogenicity of the strains and their killing capacity for *C. elegans* in the assays just described. Pathogenic bacteria like *S. pneumoniae* show the same killing effects as oral commensals like *S. mitis* and *S. oralis*. The mortality of *C. elegans* reflected solely the H_2O_2 production of the bacterial strains.

Streptococcal production of H_2O_2 has several effects in humans. It inhibits a variety of competing organisms in the upper respiratory tract (13) and causes direct oxidative damage to brain ependymal cells (9), ciliated nasal epithelium (7), and

alveolar epithelial cells (4). It is an important virulence factor in, e.g., pneumococcal colonization and host cell damage and induces apoptosis in brain cells (2). *C. elegans* is an established model for the study of apoptosis. Key components of the programmed cell death pathway are conserved between *C. elegans* and humans (17). Modification of the assay conditions might allow the use of *C. elegans* as a model to study the induction of apoptosis by pneumococci.

In conclusion, H_2O_2 production is a general factor by which streptococci can kill *C. elegans*. Our results indicate that *C. elegans* may be used as a model system to study aspects of streptococcal pathogenicity which are linked to H_2O_2—in particular, pneumococcal meningitis. Since *C. elegans* requires aerobic conditions for growth, H_2O_2-mediated killing may superimpose other killing mechanisms in catalase-negative bacteria.

We thank M. Saito for providing GAS strains SP1 and SP2.

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Editor: D. L. Burns