A Porphyromonas gingivalis haloacid dehalogenase family phosphatase interacts with human phosphoproteins and is important for invasion

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Haloacid dehalogenase (HAD) family phosphatases are widespread in prokaryotes and are generally involved in metabolic processes. Porphyromonas gingivalis, an invasive periodontal pathogen, secretes the HAD family phosphoserine phosphatase SerB653 when in contact with gingival epithelial cells. Here we characterize the structure and enzymatic activity of SerB653 and show that a SerB653 allelic replacement mutant of P. gingivalis is deficient in internalization and persistence in gingival epithelial cells. In contrast, mutation of a second HAD family serine phosphatase of P. gingivalis (SerB1170), or of a serine transporter, did not affect invasion. A pull-down assay identified GAPDH and heat-shock protein 90 as potential substrates for SerB653. Furthermore, exogenous phosphatase regulated microtubule dynamics in host cells. These data indicate that P. gingivalis has adapted a formerly metabolic enzyme to facilitate entry into host cells by modulating host cytoskeletal architecture. Our findings define a virulence-related role of a HAD family phosphatase and reveal an invasion of an important periodontal pathogen.

Periodontal diseases are among the most common infections of humans, with an estimated 5–20% of the world’s population suffering from generalized periodontitis (1). The development of periodontal disease is a multifactorial process involving interactions between the host and microorganisms that colonize the gingival sulcus. The Gram-negative anaerobe Porphyromonas gingivalis has been strongly implicated in the initiation and progression of periodontal disease and possesses a sophisticated array of virulence factors, including those that allow the bacterium to adhere to and invade host epithelial cells (2, 3). Invasion is accomplished through manipulation of host signal transduction and remodeling of cytoskeletal architecture. Moreover, P. gingivalis localizes to the perinuclear region, where it remains viable and blocks apoptosis of the infected cell (4).

The molecular mechanisms used by P. gingivalis to facilitate internalization and intracellular survival are only partially understood. A screen of proteins secreted by P. gingivalis after contact with epithelial cells revealed a putative phosphoserine phosphatase (SerB; refs. 5 and 6), corresponding to P. gingivalis ORF PG0653 (7). Based on motif searches, this protein is a member of the haloacid dehalogenase (HAD) superfamily. Although this enzyme family derives its name from the bacterial hydrolytic dehalogenases (8), the group also includes phosphomutases, ATPases, phosphotransferases, and phosphatases. Overall homology between family members is low, but all are defined by the presence of three highly conserved motifs containing five catalytic residues that form the active site. All HAD family phosphatases use aspartate as the nucleophile, form a phosphoenzyme intermediate during phosphoryl transfer, and have an absolute requirement for a divalent ion cofactor (9).

HAD family enzymes are ubiquitous, with several thousand members identified in genomic databases, but only a small number of enzymes are well studied. The few members of the family that have a defined function are associated with membrane transport, metabolism, signal transduction, and nucleic acid repair. Recently, two eukaryotic HAD phosphatases, Eyes absent and Chronophin, were characterized for their respective roles as a transcriptional cofactor (10) and a regulator of actin dynamics (11). Such reports begin to reveal the diverse functions managed by this widely spread but poorly understood group of enzymes.

This report delineates our investigations into the role of the P. gingivalis phosphoserine phosphatase during invasion of gingival epithelial cells. We used biochemical analysis to confirm this enzyme as a member of the HAD family of phosphatases. Study of allelic exchange mutants in antibiotic protection assays indicated that the SerB653, but not other HAD phosphatases or metabolic enzymes, was required for maximum invasion efficiency. Furthermore, both internalization and intracellular survival were affected by loss of this enzyme. Screens of SerB653 interactions with gingival epithelial cell extracts showed that the phosphatase may exert its effect on invasion through components of membrane vesicular transport systems, including GAPDH and microtubules. SerB653 is a previously uncharacterized HAD family phosphatase that is exploited by a prokaryote to facilitate an intracellular lifestyle.

Results

Structural Features of P. gingivalis Phosphoserine Phosphatase Genes. P. gingivalis locus PG0653 is predicted to encode a protein classified as a member of the phosphoserine phosphatase subgroup of the HAD family of hydrolases, based on the presence of three highly conserved motifs that define this group of enzymes (8). In addition, SerB PG0653 (SerB653) contains an N-terminal ACT domain motif (Fig. L4) that functions as a regulatory ligand-binding module and is commonly associated with enzymes subject to allosteric regulation (12). PG0653 also has two putative transmembrane domains and thus may be associated with the bacterial outer membrane (Fig. LB). The P. gingivalis genome encodes a second predicted HAD family SerB enzyme, PG1170. The PG0653 and PG1170 proteins are 36% identical and 61% similar in the phosphate region and are both closely related to the putative phosphoserine phosphatase encoded by Bacteriodes thetaiotaomicron VP1-5482, (71% and 41% identity, respectively) (7, 13). Sequence of the PG0653 gene from strain 33277 is identical to that of the sequenced strain W83, whereas the PG1170 ORF contained 21 nucleotide changes that resulted in five amino acid substitutions (Fig. 1C). None of the substitutions were located in the phosphatase motifs. We analyzed the two phosphoserine phosphatases in tandem, to differentiate between potential pathogenic and metabolic activities.

SerB653 Is Active on Phosphoserine and Phosphorylated Serine Peptides. To determine the substrate reactivity profiles of SerB653 and SerB PG1170 (SerB1170), purified his-tagged proteins were tested...
SerB653 on phosphoserine is consistent with allosteric regulation, with an H value of 3.7. SerB1170 is active on phosphothreonine rather than phosphoserine (190-fold) or phosphotyrosine (39-fold), indicating SerB1170 would be more accurately classified as a phosphothreonine phosphatase. Substrate preferences on phosphorylated peptides showed a similar pattern, with SerB653 most active on the phosphoserine peptide, whereas SerB1170 worked best on the phosphothreonine peptide (Fig. 2A). Although these peptides are likely not ideal substrates for the phosphatases, their utility as test substrates indicates that the active sites of SerB653 and SerB1170 are both able to accept substrates larger than amino acids.

A key characteristic of HAD phosphatases is their dependence on divalent cations as part of the active site. Addition of either the cation-displacing inhibitor sodium fluoride or EDTA to either phosphatase reaction resulted in a decrease in enzyme activity, confirming the importance of cofactors for these enzymes (Table 2). The phosphatase analog orthovanadate was also inhibitory; vanadate ion is a classic protein phosphatase inhibitor that mimics the transition state of phosphoryl-transfer reactions and competes with the substrate for interaction with the nucleophile. Vanadate inhibition indicates that the phosphatase activity of these enzymes utilizes a covalent phosphoenzyme intermediate, a feature shared by other HAD phosphatases (15, 16). Neither phosphatase was inhibited by up to 5 mM okadaic acid, an inhibitor specific for PPP family serine/threonine phosphatases. To further verify that SerB653 is a HAD-family phosphatase, site-directed mutagenesis was used to convert the predicted nucleophile aspartate 198 to asparagine. Activity of the D198N mutant enzyme was 9% of wild-type on the phosphoserine substrate (Fig. 2B), verifying the importance of the aspartate 198 residue for catalysis.

SerB Gene Replacement Mutations and Effect on Invasion Efficiency. Because the SerB653 protein, but not the SerB1170 protein, was originally recovered extracellularly in the presence of gingival epithelial cell (GEC) components (5), we hypothesized that SerB653 might play a role in the invasion process of *P. gingivalis*. *serB653* and *serB1170* single and double allelic replacement mutants were thus constructed and tested for invasion of epithelial cells. To control for the possibility that loss of serine phosphatase activity affects serine production and metabolic fitness, we also constructed a serine transporter mutant *srfT:ermF* (17). Growth curves in complex media were performed for all mutants to test for changes in cell growth or fitness. Although the double mutant *serB653::ermF*/*serB1170::tetQ* showed a slight delay in entry to log-phase, all other mutants displayed growth curves indistinguishable from the wild type (not shown). Loss of *serB653* gene product, either in a single or double mutation, significantly (*P < 0.001*) reduced invasion by *P. gingivalis* of the human immortalized gingival keratinocyte (HIGK) cell line (Fig. 3A). In contrast, loss of the phosphatase SerB1170 alone, or of the serine transporter, did not affect invasion efficiency. To ensure that the role of SerB653

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**Table 1. SerB activity on phosphorylated amino acids**

<table>
<thead>
<tr>
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<th><em>His-SerB653</em></th>
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<th><em>His-SerB1170</em></th>
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<tr>
<td></td>
<td><em>Km</em>, µM</td>
<td><em>k_cat</em> min⁻¹</td>
<td><em>k_cat/Km</em> µM⁻¹</td>
</tr>
<tr>
<td>P-Ser</td>
<td>2.041</td>
<td>1.508</td>
<td>7.3 × 10⁻¹</td>
</tr>
<tr>
<td>P-Thr</td>
<td>117</td>
<td>0.4</td>
<td>3.4 × 10⁻³</td>
</tr>
<tr>
<td>P-Tyr</td>
<td>47</td>
<td>0.03</td>
<td>6.3 × 10⁻⁴</td>
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The phosphatase activity of His-tagged fusion proteins was assayed by measuring phosphate release from phosphoserine (P-Ser), phosphothreonine (P-Thr), and phosphotyrosine (P-Tyr). *Km* and *k_cat* values were determined from substrate concentration curves analyzed with nonlinear regression.
was not strain- or cell line-specific, antibiotic protection assays were also performed with *P. gingivalis* strain W83 and its isogenic serB653 mutant and with both isogenic parent–mutant pairs in primary GEC. Similar to 33277, the W83 serB653 mutant showed a significant reduction in HIGK cell invasion efficiency compared to its parental strain (Fig. 3B). Indeed, the reduction of invasion with the W83 mutant was more pronounced than that of the 33277 mutant, possibly the consequence of the W83 strain lacking invasion effectors such as the long fimbriae (18). In GEC, the reduction in invasion by the 33277 serB653 mutant was less than in HIGK cells (Fig. 3C), probably because transformation altered signal transduction pathways involved in invasion of HIGK cells. Parent-to-mutant ratios for W83 in GEC were similar to HIGK cells. To confirm the involvement of SerB653 in the reduced invasion phenotype, we complemented the 33277 mutant with a plasmid containing the SerB653 gene (pTSerB). Additionally, the parent plasmid pTCow was conjugated into the wild-type 33277 strain and into the 33277 serB653::erm mutant; these strains were used as positive and negative controls for invasion. The presence of plasmid impeded *P. gingivalis* invasion efficiency; nonetheless, the complemented strain showed a 16-fold improvement in invasion over the mutant (Fig. 3D).

**Table 2. Inhibition of SerB activity**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>His-SerB653</th>
<th>His-SerB1170</th>
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<tr>
<td>NaF</td>
<td>5.9 mM</td>
<td>57 mM</td>
</tr>
<tr>
<td>EDTA</td>
<td>4.4 mM</td>
<td>11 mM</td>
</tr>
<tr>
<td>Na3VO4</td>
<td>1.3 mM</td>
<td>51 mM</td>
</tr>
<tr>
<td>Okadaic acid</td>
<td>&gt;5 μM</td>
<td>&gt;5 μM</td>
</tr>
</tbody>
</table>

Affect of common phosphatase inhibitors on enzyme activity. His-SerB 653 reactions were performed on phosphoserine, and His-SerB 1170 on phosphothreonine. The concentration of inhibitor required to reduce activity to 50% of wild type is shown.

**Fig. 3.** Allelic exchange mutants and gingival epithelial cell invasion. For all graphs, invasion levels are represented as total colony-forming units per 2 × 10⁵ cells. Statistical significance between invasion levels was measured by using an unpaired *t* test. *, *P* < 0.001; **, *P* < 0.05. (A) Antibiotic protection assays in HIGK cells using *P. gingivalis* strain 33277 and the isogenic mutants described in Materials and Methods. (B) Invasion of HIGK cells with the complemented SerB653 mutant. Statistical significance is between mutant and complemented strains.

SerB653 Mutation Results in a Delay in Cell Penetration but Not Adhesion. Invasion of eukaryotic host cells by bacterial pathogens is a complex process, requiring bacterial attachment, penetration, and intracellular survival. The antibiotic protection assay reports the collective outcome of these processes. To identify the stage of invasion affected by the serB653 mutation, an adherence assay and invasion time course were performed. No significant difference in adherence to HIGK cells between the *P. gingivalis* wild-type strains 33277 and W83 as compared to their isogenic mutants was observed (Fig. 4A). For the invasion time course, we used fluorescent microscopy to quantitate the levels of intracellular *P. gingivalis*. After 10 min of bacterial interaction with HIGK cells, both parental and mutant strains showed similar low levels of invasion (Fig. 4B). However, invasion by the wild type was complete within 30 min, whereas the mutant required 2 hr to complete the invasion process. The timing of the wild-type invasion is consistent with previous studies in primary GECs, in...
which *P. gingivalis* was shown to complete invasion within 20 min (20). These results indicate that the SerB653 mutant deficiency is at the stage of host cell internalization. Additionally, although fluorescent microscopy reveals the presence of the *P. gingivalis* SerB653 mutant at wild-type invasion levels after 2 hr, the antibiotic protection assay demonstrates there are less viable mutant cells in the host at the same time point. Thus, in addition to the deficiency in internalization, the SerB653 mutant is compromised in its ability to survive inside host cells. Experiments are ongoing to investigate interactions between the SerB mutant and intracellular microbial sensing components such as nucleotide-binding oligomerization domain (NOD) proteins (21).

**Interactions Between SerB653 and Host Proteins.** To identify epithelial cell proteins that might interact with SerB653 during the invasion process, we used a bead-based pull-down kit for detecting protein prey with his-tagged bait. Two proteins (85 and 37 kDa) from HIGK cell extracts were identified by the pull-down assay (Fig. 5A). Mass spectrometry identified four peptides from the 85-kDa band (Fig. 5B), which matched mammalian heat-shock protein 90 (HSP90). Five peptides sequenced from the 37-kDa protein (Fig. 5C) identified it as human GAPDH. Each of these proteins is known to be phosphorylated on serine residues and thus able to serve as targets for serine phosphatases (22, 23). Human HSP90 is a chaperone with a set of ~100 protein clients, many of which are involved in signal transduction pathways (24). Although the role of HSP90 phosphorylation is not completely understood, it has been shown in several cases to modulate the affinity of chaperone/client interactions (25). GAPDH is primarily known for its role as a glycolytic enzyme, but accumulating evidence suggests that this enzyme is involved in a variety of activities unrelated to energy production, including membrane fusion, microtubule bundling, DNA repair, and apoptosis (26). GAPDH has been linked to the recruitment of microtubules to membranes during vesicular trafficking, and it has been hypothesized that phosphorylation regulates the interaction of GAPDH with tubulin during this process (27). Because microtubule activity is known to be required for optimal *P. gingivalis* invasion (2, 3, 28), and because microtubules play an important role in cell internalization for other invasive pathogens (29), we decided to further investigate the possible link between SerB653 and tubulin dynamics.

**SerB Enzyme Alters Microtubule Dynamics During Invasion.** Because SerB653 can be released into the external milieu by *P. gingivalis* (5), the response of HIGK microtubules to extracellular SerB653 was examined. Fluorescent microscopy (Fig. 6) revealed that after 30 min of incubation, cells exposed to SerB653 phosphatase show a striking rearrangement of microtubules to the cell surface compared to control cells. This effect was further exaggerated at the 2-hr time point, where some cells with microtubule rearrangements also show significant changes in cell morphology. Microtubule remodeling by SerB653 may provide the mechanism by which the enzyme can modulate *P. gingivalis* penetration and is consistent with GAPDH acting as a SerB653 substrate. SerB653, however, likely has multiple effects on epithelial cell signal transduction pathways, and the precise relationship among SerB653, GAPDH, and tubulin is undergoing investigation in our laboratory.

**Discussion**

Tightly regulated protein phosphorylation and dephosphorylation are an important means of signal transduction in both prokaryotes and eukaryotes. In prokaryotes, protein phosphorylation is mediated primarily by histidine kinases; however, serine/threonine kinases and phosphatases have also been described, and these enzymes are important for virulence in *Yersinia pseudotuberculosis*, *Pseudomonas aeruginosa*, *Streptococcus agalactiae*, and *Listeria monocytogenes* (30, 31). Moreover, *Salmonella*, *Shigella*, and *Yersinia* use type III secretion machinery to translocate kinases and phosphatases directly into host cells, where they can interfere with host-cell signal transduction,
The best-defined phosphoserine phosphatases belong to proteobacteria taxonomic group. These enzymes are responsible for the biosynthesis of serine from 3-phosphoserine and as such are part of the serine/threonine biosynthetic pathway. As an asaccharolytic organism, *P. gingivalis* acquires energy by uptake and catabolism of small peptides; biosynthesis of serine is therefore likely to be unnecessary under physiological growth conditions. The lack of pressure on this enzyme for fitness would make it eligible to develop new functions unrelated to biosynthesis or metabolism.

The SerB653 mutant exhibits a normal growth rate in laboratory medium but a reduced invasion efficiency for gingival epithelial cells. Moreover, intracellular survival of the mutant is also attenuated. Hence, SerB is a multifunctional invasin of *P. gingivalis* that is involved both in entry and intracellular persistence. In coprecipitation experiments, purified recombinant SerB653 pulled down GAPDH and HSP90. Serine phosphorylation is thought to regulate microtubule/GAPDH interactions in vesicular trafficking (23, 27, 34, 35), and interestingly this model predicts the presence of an unknown eukaryotic phosphatase as part of the regulatory system. Thus, our working hypothesis is that the *P. gingivalis* SerB653 HAD-type phosphatase may be emulating a host enzyme to modulate the recruitment of microtubules to host cell membranes. Consistent with this, exogenous SerB653 recruits microtubules to the surface in epithelial cells.

The role played by the SerB653 interaction with HSP90 remains to be established. It is possible that modulation of HSP90 phosphorylation might change the subset of clients activated by the chaperone. Alternatively, the interaction between these two proteins might be driven by the heat-shock protein, which is known to refold proteins that are insoluble or coaggregated (36).

In conclusion, analysis of *P. gingivalis* SerB653 has led to the discovery of a previously undescribed role for a HAD family phosphatase. SerB653 activity impacts microtubule dynamics and is required for optimal invasion and intracellular survival of the organism. The challenges now are to elucidate the mechanistic basis of enzyme secretion, internalization, and intracellular activity. Such investigations will provide new insights into the multifactorial virulence mechanisms of *P. gingivalis*.

**Materials and Methods**

**Bacterial Strains and Cell Culture.** *P. gingivalis* strains were grown anaerobically at 37°C in trypticase–soy medium supplemented with hemin and menadione. *Escherichia coli* strains were grown in LB media aerobically at 37°C. The *E. coli* strain DH5α (Stratagene) was used as a host for cloning and plasmid purification. *E. coli* strain TunerDE3 (Novagen) was used to purify the His-tagged phosphatases. Primary cultures of GEC were obtained from gingival explants and were maintained in culture as described (37). Human immortalized gingival keratinocytes were obtained from D. Oda (University of Washington, Seattle, WA) and cultured as described (38).

**Plasmid Construction and Protein Purification.** The phosphoserine phosphatase ORFs (PG0653 and PG1170, www.tigr.org) were PCR-amplified from genomic DNA of *P. gingivalis* 33277 by using *Pfu* polymerase (Stratagene) and cloned into the plasmid pET30b (Novagen), resulting in plasmids pET30b-653 and pET30b-1170. These plasmids were sequenced to confirm the construct. A phosphatase-deficient mutant of the 653 enzyme was created by site-directed mutagenesis, using primers to alter the catalytic aspartate 198 to asparagine. Proteins were purified by using a BioLogic DuoFlow chromatography system loaded with an IMAC column (Bio-Rad). Purity was >90%, as determined by SDS/PAGE Coomassie staining. The complementation plasmid pTserB653 was created by cloning the SerB653 wild-type gene into the *E. coli*–*Bacteroides* shuttle vector pTCow (39), and it was subsequently conjugated into the PG0653::ermF mutant. (See Supporting Text and Fig. 8, which are published as supporting information on the PNAS web site, for additional details.)

**Construction of Insertion and Allelic Exchange Mutants.** A *P. gingivalis* mutant in the serine uptake transporter sstT (17) was created by cloning a 600-bp internal fragment of the gene into the *P. gingivalis* suicide vector pVA3000 (40) and conjugation of the construct into strain 33277. The mutant was confirmed by Southern hybridization. Allelic exchange mutants in PG0653 and PG1170 were created by cloning ~2-kb gene fragments into pUC19, then inserting either ermF (PG0653) or tetQ (PG1170) into the ORF. The resulting constructs were digested with ScaI and the linear DNA electroporated into *P. gingivalis* (41). Transformants were selected on erythromycin or tetracycline and confirmed by PCR and Southern hybridization. A double mutant in both 653 and 1170 was created.
by transforming the confirmed PG0653::ermF mutant with the pUC19-1170 linear construct.

Phosphatase Enzyme Assays. Enzymes were tested against a panel of divalent cations to determine the optimal cofactor for activity. SerB653 showed optimal activity in MgCl$_2$ or MnCl$_2$, whereas SerB1170 was most active in MgCl$_2$ or CoCl$_2$. Phosphatase assays were performed in 25- to 50-$\mu$L reactions at 30°C, in 50 mM Tris (pH 7.5), with 5 mM MgCl$_2$. Reactions with amino acid substrates were incubated at 30°C for 5–15 min, and phosphate release was detected with malachite green dye according the manufacturer’s directions (Ser-Thr Phosphatase Kit, Promega). Enzyme-to-substrate ratios were >1:100. $K_m$ and $k_{cat}$ values were calculated from substrate concentration curves by using nonlinear regression of at least two replicates for each concentration point (GRAPHPAD PRISM, GraphPad, San Diego). The peptide phosphatase reactions were incubated for 1 hr at 30°C.

Protein Interaction Assay. Pull-down assays were performed to detect potential interactions between the SerB653 phosphatase and eukaryotic proteins by using the ProFound Pull-Down Protein Interaction Kit (Pierce), as described by the manufacturer (see Supporting Text). HIGK cell protein extracts were prepared from cells pretreated with calyculin A for 30 min. Pull-down controls were His-SerB653 D198N and His-tag coated beads incubated with HIGK protein extract. Sample and controls were run on a 10% SDS/PAGE gel (42), and bands unique to the sample were excised. Protein bands were identified by using MALDI-TOF mass spectrometry by the University of Florida Protein Core Facility.

Adherence Assays. Adherence assays were performed on formalin-fixed HIGK cells, as described (43), and adherent *P. gingivalis* detected by using specific antibodies (44) (see Supporting Text).

Antibiotic Protection Invasion Assay. Antibiotic protection assays were performed in 24-well cell culture plates containing $\sim$2.5 x $10^5$ HIGK cells or primary GEC (see Supporting Text).

Fluorescent Microscopy. Invasion time-course assays were performed in four-well chambered slides containing 90% confluent HIGK cells. *P. gingivalis* cells were added at a multiplicity of infection of 100 and incubated for the appropriate time. Wells were washed three times with $1\times$TBS + 0.1% Triton X-100 to remove external bacteria, then invaded cells were fixed with ice-cold methanol. Internalized bacteria were detected with polyclonal rabbit anti-*P. gingivalis* antibody and FITC-conjugated anti-rabbit secondary antibody. Images were taken by using a Zeiss Axioplan 2 microscope equipped with a RGB Spot camera by using the FITC filter set and a $\times 60$ lens. Fluorescent intensity of images was quantitated by using Mophometrics software (Imageing Research, St. Catherine’s, ON, Canada). Grain-count values are the results of three images containing an average of 300 HIGK cells. To visualize microtubule dynamics, purified enzyme (100 $\mu$g) was added to wells containing HIGK cells. Cells were washed at appropriate time points three times with $1\times$ TBS and fixed with cold methanol. Cells were incubated with $\alpha$-tubulin monoclonal antibodies (Acris Antibodies, Hiddenden, Germany) and detected with anti-mouse secondary antibody conjugated to Texas red (Ab-Cam, Cambridge, MA). Images were taken at $\times 60$ with a Zeiss Axioplan 2 microscope.

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